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13. ABSTRACT (Maximum 200 Words)

It has been well documented that normal prostate contains the highest zinc levels among all other soft tissues in the body. However, once the normal prostate cells transform to malignant cells, the zinc levels decreased significantly. We have demonstrated two major functions of zinc in prostate: metabolic effect and growth effect. This proposal aimed to establish that the prostatic tumor cells are citrate oxidizing cells; and to demonstrate that the alteration of zinc accumulation will alter the citrate production and eventually inhibit the tumor cell growth. The third year study was focused on: 1) to continue the study of the zinc effect on prostate tumorigenicity *in vivo*; 2) to study the mechanism of zinc induced PC-3 cell apoptosis; 3) to extend our understanding of zinc effect on the relationship of metabolism and growth in prostate tumor cell, the effect of zinc on prostate TRAMP cells was studied.

Over the past three years with DOD grant support, we have published 6 papers in peer reviewed journals and prepared 2 manuscripts for submission. We have also presented our research data with 14 published abstracts in academic meetings (see Appendix).

14. SUBJECT TERMS
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Table of Contents

Introduction

It has been well documented that normal prostate contains the highest zinc levels among all other soft tissues in the body. However, once the normal prostate cells transform to malignant cells, the zinc levels decreased significantly. We have demonstrated two major functions of zinc in prostate: metabolic effect and growth effect. This proposal aimed to establish that the prostatic tumor cells are citrate oxidizing cells; and to demonstrate that the alteration of zinc accumulation will alter the citrate production and eventually inhibit the tumor cell growth. This third year study was focused on: 1) to continue the study of the zinc effect on the prostate tumorigenicity *in vivo*; 2) to study the mechanism of zinc induced PC-3 cell apoptosis; 3) to extend our understanding of the zinc effect on the relationship of metabolism and growth in prostate tumor cells, and the effect of zinc on prostate TRAMP cells was studied.

Over the past three years, with DOD grant support, we have published 6 papers in peer reviewed journals and prepared 2 manuscripts for submission. We have also presented our research data with 14 published abstracts in academic meetings (see Appendix).

In this report we will address the research progress in the third year period. For the first and second year research summaries, please refer to our previous reports.

Body

The research progress and accomplishments associated with our proposed tasks are reported as following:

Experiments and Results:

1. To study the zinc effect on tumorigenicity, and on the normal rat prostate *in vivo.*

We continued the study of the zinc effect on prostate tumorigenicity in nude mice as we reported last year. The detailed results were published (Feng et al 2004); please see a reprint in the Appendix.

The results from our *in vivo* study indicated that *in vivo* treatment of zinc increased zinc accumulation in the tumor tissues and inhibited PC-3 cell derived tumor growth. This inhibitory effect of zinc may result from zinc-induced apoptosis through the regulation of the mitochondrial apoptosis pathway. If zinc can induce apoptosis in malignant cell derived prostate tumor, a question of what is the effect of zinc treatment on normal prostate cells *in vivo* has been raised.

To address this important issue, we studied the effect of zinc on normal rat prostate *in vivo.* The **objectives** of this study are to investigate the effect of zinc treatment on 1) the zinc accumulations in the prostate tissues, 2) the levels of citrate production, 3) the growth (apoptosis) of the prostate tissues, 4) the effect of zinc on rat body weight. **Methods**: Normal adult rats were treated with zinc 5mg/kg body weight (*s.c.*) twice a

week for five weeks, and the animals receiving saline only were used as controls. Prostate tissues were then dissected and used for the experiments. **Results**: The results showed that zinc treatment significantly increased the zinc level in lateral lobe of prostate tissues compared to the control, but no change of zinc levels in both ventral and dorsal lobes was observed (Fig. 1). In corroboration with zinc levels, a significant increase in citrate production was found in lateral prostate tissues from rats treated with zinc comparing with the control animals. No significant changes of citrate levels were detected in ventral and dorsal tissues (Fig. 2).

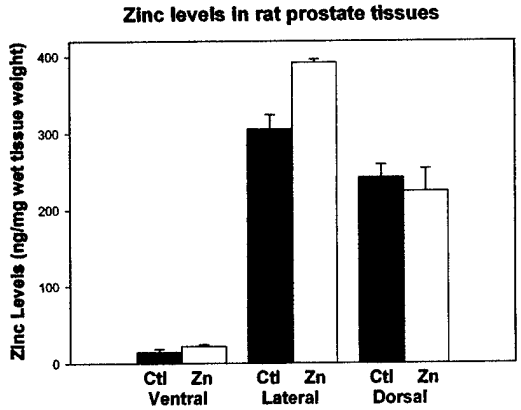


Fig. 1. Zinc levels in rat prostate tissues.

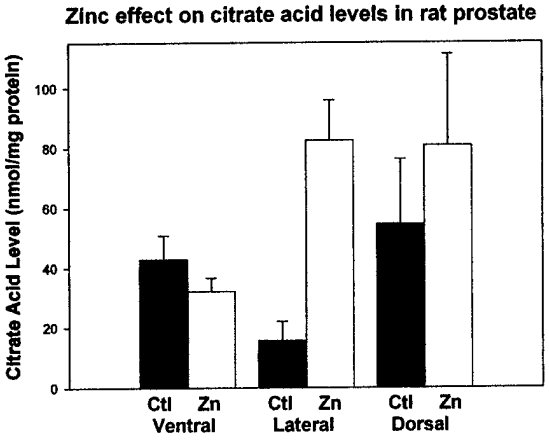
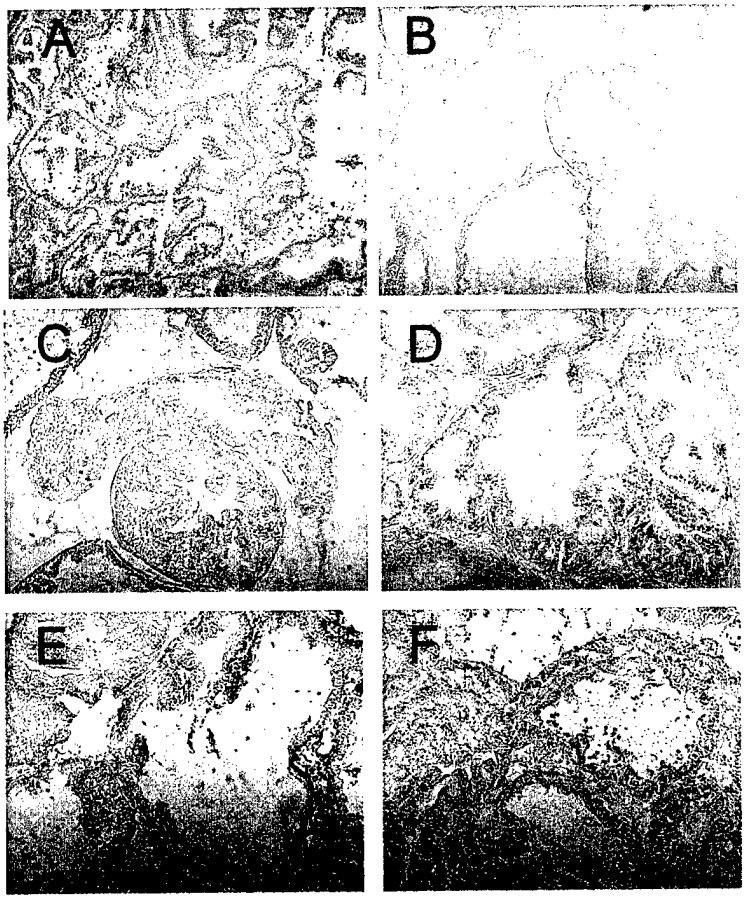
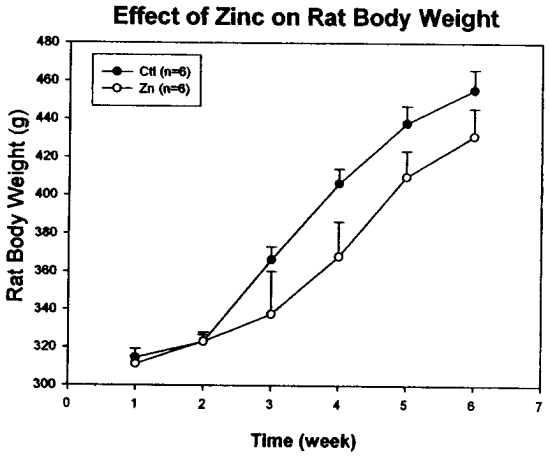


Fig. 2 Zinc effect on citrate levels.



The effect of zinc on the growth of rat normal prostate tissue was studied by TUNEL assay (Fig. 3). The results showed that zinc treatment induced prostatic cell apoptosis, observed in the position close to the basal membranes of rat lateral prostate tissues, compared with the controls. However, no significant difference of cell apoptosis appeared in rat ventral prostate treated with zinc.

Fig. 3 TUNEL assay of rat prostate.
A: Positive control of the assay
B: Negative control of the assay
C: Control of lateral prostate
D: Zinc treated Lateral Prostate
E: Control of Ventral prostate
F: Zinc treated Ventral Prostate



To study the toxicity of zinc treatment (4mg/kg) on experimental animals, rat body weight was measured weekly. The results showed zinc treatment slightly reduced the body weight of the rats receiving zinc, although there were no significant differences detected between the two groups.

Fig.4 Rat body weights.

Conclusions: Zinc treatment at the dosage of 4 mg/kg body weight for twice a week for 4 weeks increases zinc accumulation and citrate production in prostate lateral lobes; and induces a minor degree of apoptosis in normal rat lateral prostate tissues. The dosage and toxicity of zinc need to be further studied.

2. To study the mechanisms of zinc induced PC-3 cell apoptosis.

In selective prostate cells, such as PC-3 cells, zinc treatment (maximum 15uM) induces mitochondrial apoptosis. This is due to a direct action of zinc on mitochondria that causes the release of cytochrome c followed by the caspase cascade leading to nuclear DNA fragmentation and cell apoptosis. The mechanism by which zinc induces mitochondrial release of cyt c was studied. The results showed that zinc treatment of PC-3 cells or their mitochondria results in an increase in the mitochondrial insertion and polymerization of Bax indicative of pore formation in mitochondrial membrane. In addition, exposure of the cells to zinc increases the cellular level of Bax and the Bax/Bcl-2 ratio, which suggests the effect of zinc favors to promote cell apoptosis. These effects are also evident in xenograft PC-3 tumor cells, when the host animals were treated with zinc.

These studies have been completed and for the details please see our included manuscript entitled "The involvement of Bax in zinc-induced mitochondrial apoptosis in prostate cells" in the Appedix.

3. To extend our understanding of the zinc effect on the relationship of metabolism and growth in prostate tumor cells, the effect of zinc on prostate TRAMP cells was studied.

We studied the effect of zinc on both PC-3 and LNCaP cells using cell cultures and tumor tissues derived from these cells. However, the tumor tissues were xenograft tissues, which challenge us to consider future studies of zinc effect on prostate in transgenic animal models. One strength of using transgenic models is that in these animals, cancer arises from normal cells in their natural tissue microenvironment and through multiple

stages, as it does in human cancer. For prostate cancer, one such model is TRAMP. Before using the TRAMP animal model to extend our study of the zinc effect on prostate, we characterized TRAMP cells (ATCC), a cell line derived from TRAMP animal prostate cancer tissue. The results are shown below.

A. Zinc inhibits TRAMP cell growth in serum free medium.

TRAMP cell were cultured in modified DMEM medium. After 24 hrs in serum free medium, the cells were treated with different concentrations of zinc as indicated in Fig. 5 for 24 hrs. Then the cells were collected and counted. The independent experiment was repeated 3 times. Results showed that zinc inhibited TRAMP cell growth in a dose-dependent manner, and 75% inhibition of the cell growth was observed by using zinc (1000ng/ml).

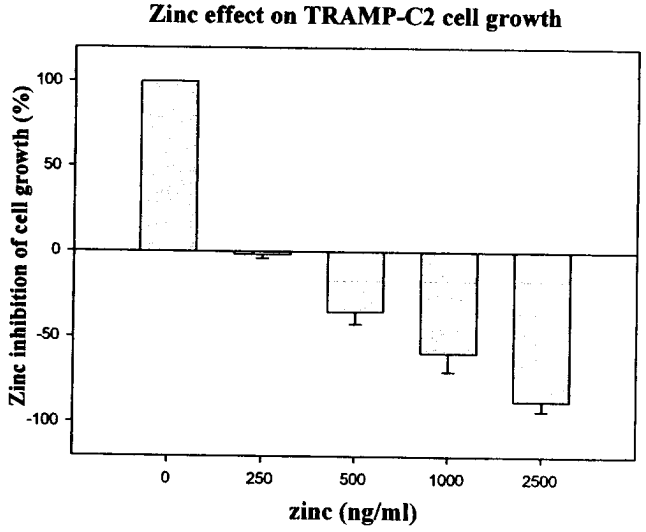
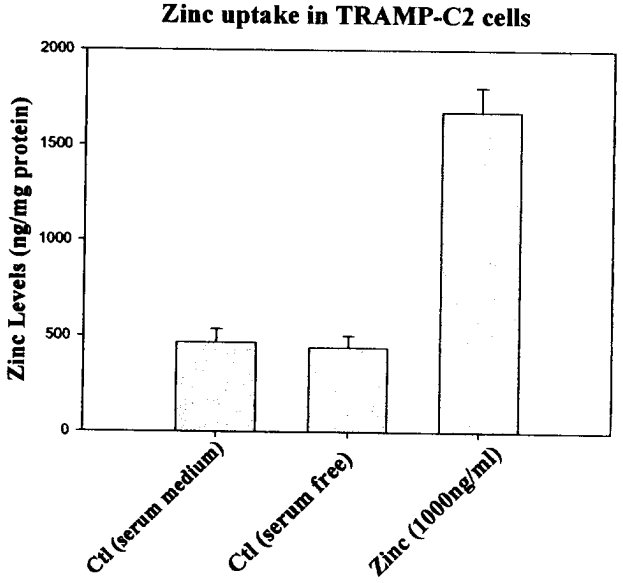


Fig.5 Zinc inhibition of TRAMP cell growth.

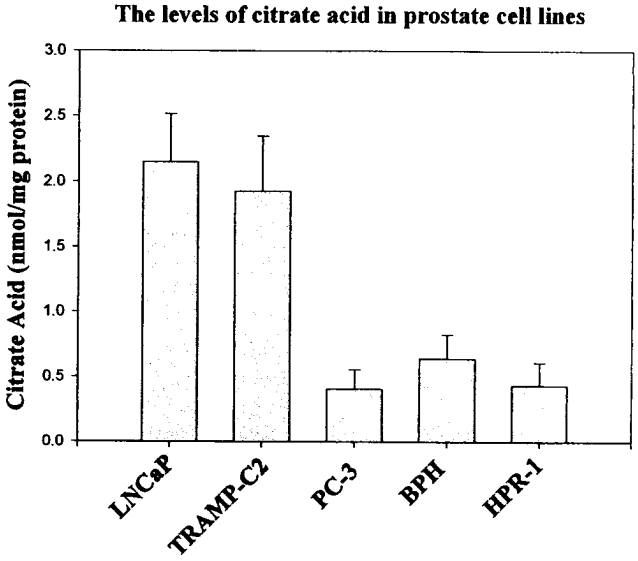
B. Zinc uptake in TRAMP cells.



TRAMP cells were treated with/without zinc (1000ng/ml) for 3 hrs. The cellular zinc levels were measured by atomic absorption method. The results showed the accumulation of zinc in TRAMP cells compared those of the controls. Serum in cell culture medium does not appear to have impact on cellular zinc accumulation.

Fig. 6 Zinc uptake in TRAMP cells.

C. TRAMP cells are citrate producing cells.

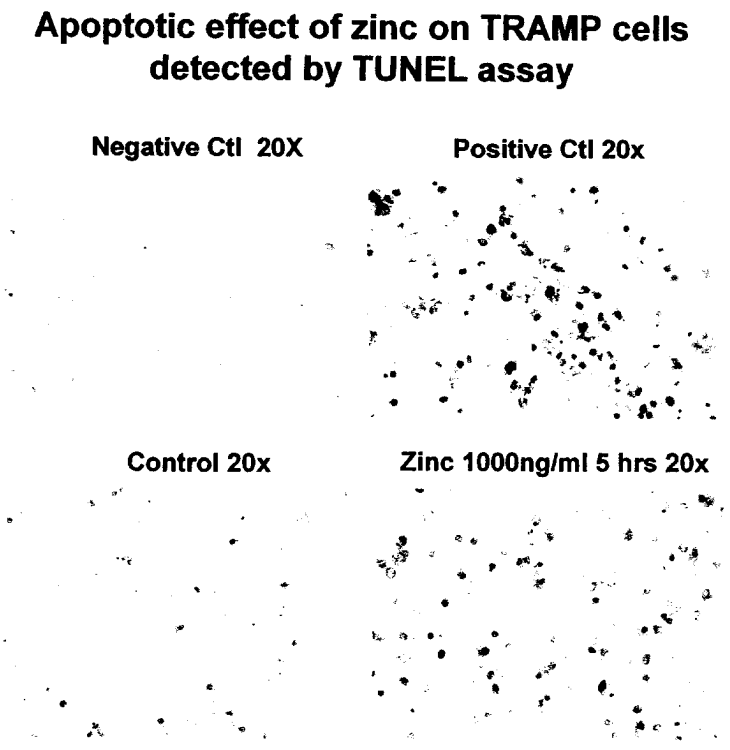


It has been demonstrated that LNCaP cells are citrate producing cells, but PC-3 cells are not. We compared TRAMP cells with cells we have studied previously shown in Fig. 7. The results indicated that citrate acid levels in TRAMP cells are similar to those in LNCaP cells, which suggests that TRAMP cells employ similar metabolic pathways as LNCaP cells do.

Fig. 7. Citrate acid levels in TRAMP cells.

D. TUNEL assay

To further study the growth inhibitory effect of zinc on TRAMP cells, TUNEL assay was used to identify cell apoptosis. TRAMP cells were cultured at the same conditions as the growth experiments (in A), and treated with or without zinc (1000ng/ml) for 5 hrs. The cells were then collected onto slides utilizing a Cytospin; the cells were fixed and further characterized by TUNEL assay conducted with the standard manufacturer's protocol.

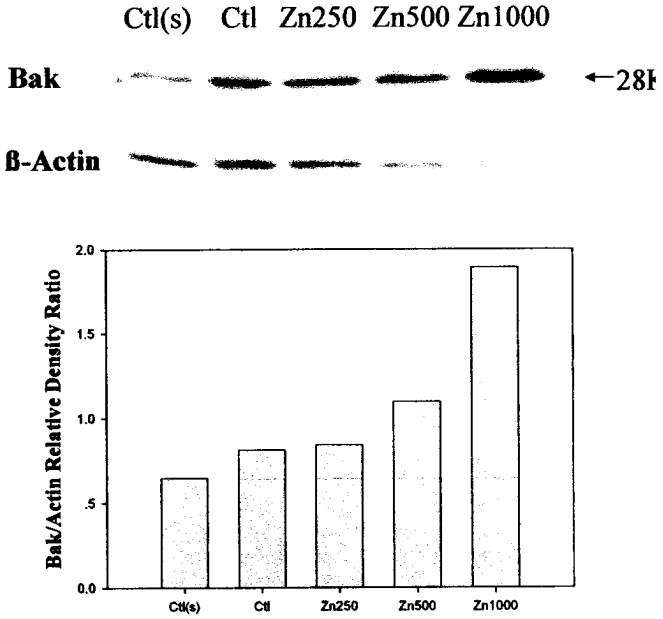


Results showed that zinc treatment of TRAMP cells induced significant increase of TRAMP cell apoptosis shown as dark brown stained apoptotic bodies, compared to the control (Fig. 8 Lower panel). The top panel shows the positive and negative controls for the assay. Our results demonstrated that the inhibitory effect of zinc on TRAMP cell growth is due to (or partially due to) zinc induced cell apoptosis.

Fig. 8 Apoptotic effect of zinc on TRAMP cells detected by TUNEL assay.

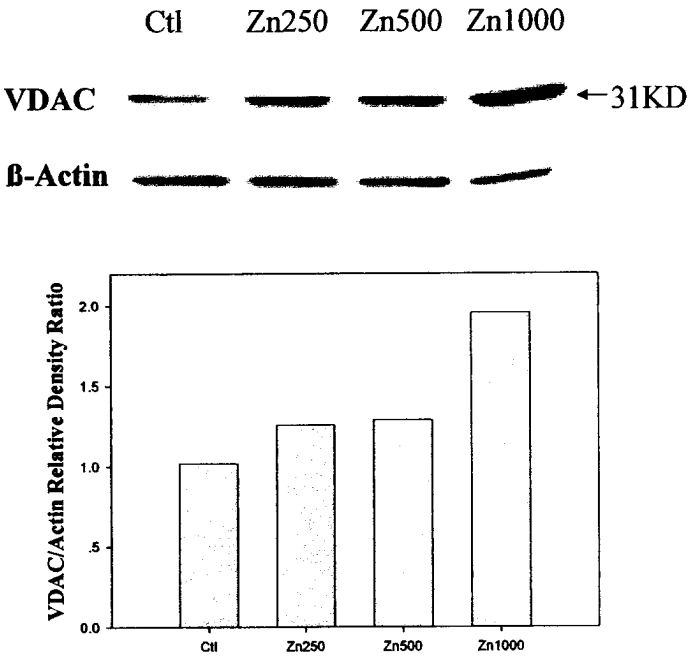
E. The mechanisms of zinc induced TRAMP cell apoptosis.

To further understand the mechanisms of zinc induced apoptosis in TRAMP cells, we determined the effect of zinc on cellular levels of pro-apoptotic factors which are associated with the mitochondrial apoptosis pathway. Western blot analysis was employed to analyze the changes of Bak and VDAC proteins in the cells treated with or without zinc.



Western blot data (Fig.9) showed that zinc treatment elevated Bak level by two fold compared with the controls. However, there was no effect of zinc on Bax (data not shown), which was shown as a major apoptotic protein in PC-3 cells (see in Exp. 2 above).

Fig. 9 Zinc increased Bak level in TRAMP cells.



A 30% increase of VDAC levels induced by zinc treatment (1000ng/ml) in TRAMP cells was detected by Western blot.

Fig.10 Zinc increased VDAC level in TRAMP cells.

Key Research Accomplishments (Three years)

- Established the optimal conditions for metabolic assays and zinc atomic absorption method.
- Established tumorigenic animal models in nude and SKID mice with PC-3 and LNCaP cells.
- Studied zinc effect on tumorigenicity using ALZET pumps.
- Studied the zinc effect on tumorigenicity in PC-3 cell induced tumors using ALZET pumps.
- Characterization of ZIP1 over expressed PC-3 cells
- Study the zinc effect on tumorigenicity in ZIP1 over expressed PC-3 cells
- To establish ZIP1 over expressed LNCaP cells
- Studied the zinc effect on the prostate tumorigenicity *in vivo*
- Studied the zinc effect on the normal rat prostate tissues *in vivo*
- Studied the mechanisms of induced PC-3 cell apoptosis
- Investigated the effect of zinc on prostate TRAMP cells

Reportable Outcomes:

Publications

1. **P. Feng**, T-L Li, Z-X Guan, R.B. Franklin, L.C. Costello Direct effect of zinc on mitochondrial apoptogenesis in prostate cells. **The Prostate** 52:311-318, 2002

2. RB Franklin, J Ma, J Zou, BI Kukoyi, **P Feng**, CL Costello. hZIP1 is a major zinc uptake transpoter for the accumulation of zinc in prostate cells. J. of Inorganic Biochemistry, 96:435-442, 2003

3. **P. Feng**, TL Li, ZX Guan, RB Franklin, LC Costello Effect of zinc on prostatic tumorigenicity in nude mice. Annals of the New York Academy of Sciences, 1010:316- 320, 2003

4. ZX Guan, B. Kukoyi, **P. Feng**, M.C. Kennedy, R.B. Franklin, L.C. Costello. Kinetic identification of a mitochondrial zinc uptake transport process in prostate cells. J. Inorganic Biochem. 97:199-206, 2003

5. LC Costello, ZX Guan, RB Franklin, and **P Feng.** Metallothionein can function as a chaperone for zinc uptake transport into prostate and liver mitochondria J. Inorganic Biochem 98:664-666, 2004

6. LC Costello, **P Feng,** B Milon, M Tan, RB Franklin. The role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve. Prostate Cancer and Prostatic Disease 7:111-117, 2004

7. **P Feng,** TL Li, ZX Guan, RB Franklin, LC Costello. The involvement of Bax in zinc-induced mitochondrial apoptogenesis in prostate cells. (Submitted, 2004)

8. ZX Guan, TL Li, RB Franklin, LC Costello, and **P Feng**. MEtallothiomein-1 and -2 gene expression and mitochondrial zinc uptake from Zn7-MT in prostate epithelial cells. (Prepared for Submission, 2004)

Abstracts

1. **P. Feng**, TL Li, ZX Guan, RB Franklin, LC Costello Effect of zinc on prostatic mitochondrial apoptogenesis in a cell-free system. p415a. American Society for Cell Biology Annual Meeting Abstract Book 2001

2. **P. Feng**, TL Li, ZX Guan, RB Franklin, LC Costello Zinc Inhibits Prostate cell growth. p18. In Press Book, American Society for Cell Biology, 2001

3. RB Franklin, J Ma, J Zou, **P Feng** and LC Costello Over expression of hZIP1 zinc transporter inhibits growth of PC-3 cells. Society for Basic Urologic Research/European Society of Urologic Research Symposium 2001

4. **P. Feng**, TL. Li, ZX. Guan, R.B. Franklin, L.C. Costello Effect of prostatic cells and zinc treatment on the development of osteoblast cells in a co-culture system. Society for Basic Urologic Research/European Society of Urologic Research Symposium 2001

5. ZX. Guan, TL. Li, R.B. Franklin, L.C. Costello and **P. Feng** Study of Metallothionein-1/2 Gene Expression and Mitochondrial Distribution in Prostate Epithelial Cells. 24th Annual Graduate Research Conference of The Graduate Student Associations at the University of Maryland Baltimore Graduate School, April 19, 2002

6. R.B. Franklin, **P. Feng**, L.C. Costello Mechanism of Zinc Accumulation in Prostate and its Effect on Prostate Cell Growth. Molecular Mechanisms as Targets in Prostatic Diseases, Liverpoos, UK, Sep. 17-20, 2002

7. RB Franklin, **P Feng**, LC Costello Regulation and mechanism of zinc accumulation in prostate. Zinc Signals 2002, Grand Cayman, BWI, April 2002

8. **P. Feng**, TL Li, ZX Guan, RB Franklin, LC Costello Effect of zinc on prostatic tumorigenicity in nude mice Poster presentation in the meeting of "APOPTOSIS 2003 From signaling pathway to therapeutic tools", Luxembourg, 2003

9. Franklin, R.B. , Z, Guan, B. Milon, **P. Feng** and L.C. Costello Mechanism of zinc transport in prostate cancer cells. Zinc Signals 2003, Grand Cayman, BWI, 2003

10. **P Feng,** RB Franklin, LC Costello Zinc induction of mitochondrial apoptogenesis in prostate cells. Oral presentation in the 48th Annual Meeting of Biophysical

Society, Baltiomre, Maryland 2004

11. ZX Guan, TL Li, RB Franklin, LC Costello, **P Feng** Metallothionein (MT)-1 and 2 gene expression and mitochondrial zinc uptake from Zn7-MT in prostate epithelial cells. Poster presentation in FASEB 2004 meeting, Washington DC, 2004

12. **P Feng,** TL Li, RB Franklin, LC Costello The involvement of Bax in zinc-induced mitochondrial apoptosis in prostate cells. Oral presentation of Zinc signals 2004, Aarhus, Denmark, 2004

13. Beatrice Milon, Qin Wu, Leslie C. Costello, **Pei Feng**, Renty B. Franklin Site Directed Mutagenesis of the Histidine 160 in hZip1 Zinc Signals 2004, Aarhus, Denmark, June 2004

14. Renty B. Franklin, Zhixin Guan, Pei Feng, Leslie C. Costello. Evidence for a mitochondrial zinc uptake transporter in prostate epithelial cells. Zinc Signals 2004, Aarhus, Denmark, June 2004

Conclusions:

With the support of this grant, the research project generated new information which was presented in our publications, meeting presentations and the communications with other scientists who are working in the field of prostate cancer research. The progress obtained from the second year study of this project is very promising as indicated in the research data presented in this report. It is very important to do the ***in vivo*** study on the mechanisms of the **zinc effect** on metabolism, which have a direct link to tumorigenicity in prostate. Although the ***in vivo*** study is generally more difficult than the ***in vitro*** study, the results will provide new insight to the pathogenesis of prostate cancer, eventually leading to a new clinical therapeutic method for prostate cancer.

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1. Gupta S., Ahmad N., Marengo SR, MacLennan GT, Grrenberg NM, Mukhtar H. Chemoprevention of prostate carcinogenesis by a-Difluoromethylornithine in TRAMP mice. Cancer Res. 60:5125-5133, 2000

2. Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM. Prostate cancer in a transgenic mouse. Proc. Natl. Acad. Sci. USA 92:3439-3443, 1995

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Effect of Zinc on Prostatic Tumorigenicity in Nude Mice

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ABSTRACT: **Prostate epithelial cells accumulate the highest zinc levels of any cells in the body. Evidence indicates that zinc plays critical roles in the normal function and pathology of the prostate gland. We have identified two important effects of zinc in the prostate epithelial cells: the inhibition of m-aconitase and the induction of mitochondrial apoptogenesis. However, at the present time, the effects of zinc on prostatic cells in *in vivo* conditions have not yet been reported. The objectives of this *in vivo* study were to investigate the effect of zinc on: tumorogenicity in nude mice, zinc accumulation in tumor tissues, and the levels of mitochondrial membrane permeability related proteins, Bax/Bcl-2. A tumorigenicity animal model was established using male nude mice (4–6 weeks old) with inoculation of PC-3 cells (5–10$\times 10^6$/mL) prepared in 10% Matrigel. The mice were treated with zinc by ALZET osmotic pumps (Durect Corporation), with a releasing rate of 0.25 μl/h for 28 days. Zinc concentrations of the tumor tissues were determined by Atomic Absorption Spectrophotometer method. Frozen sections of tumor tissues were prepared for TUNEL assay. The levels of Bax and Bcl-2 in the tumor tissues were determined by Western blot analyses. Our study demonstrated that *in vivo* treatment of zinc increased zinc accumulation and citrate production in PC-3 cell induced tumor tissues and inhibited tumor growth. The inhibitory effect of zinc appears to result from zinc-induced apoptosis by regulation of mitochondrial membrane permeability-related Bax/Bcl-2 proteins.**

KEYWORDS: **prostate; zinc; tumor; apoptosis; Bcl-2/Bax**

Prostate epithelial cells accumulate the highest zinc levels of any cells in the body. However, malignant prostate cells have lost this ability; and evidence indicates that zinc plays critical roles in the normal function and pathology of the prostate gland. We have identified two important effects of zinc in the prostate epithelial cells: the inhibition of *m*-aconitase and the induction of mitochondrial apoptogenesis.[1,2,3] In a normal prostate, zinc inhibition of *m*-aconitase activity results in suppressing citrate oxidation, and increases citrate production. Our recent studies demonstrate that zinc induces mitochondrial apoptogenesis in cultured PC-3 (human prostatic malignant cell line) and BPH (benign prostatic hyperplasia) cells. Furthermore, exposure of mi-

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tochondria isolated from these cells to zinc results in the release of cytochrome *c* and redistribution of Bax/Bcl-2 on mitochondrial membranes (Bax/Bcl-2 data unpublished). However, the effects of zinc on prostatic cells in *in vivo* conditions have not yet been reported.

The objectives of this *in vivo* study were to investigate the effect of zinc on tumorogenicity in nude mice, zinc accumulation in tumor tissues, and the levels of mitochondrial membrane permeability related proteins, Bax/Bcl-2.

The experiments were carried out using male nude mice (4-6 weeks old) as a tumorigenicity animal model. The mice were housed in a pathogen-free environment under controlled light and humidity. Tumors were established by inoculation of PC-3 cells ($5–10\times10^6$/mL) prepared in 10% Matrigel and using 0.1 mL for each injection (s.c.) at both flanks of the animals. The size of tumors was measured weekly. Zinc treatment was given by ALZET osmotic pumps (Durect Corporation), with a releasing rate of 0.25 μL/h for 28 days. The pumps were filled with PBS (control), zinc sulfate 5 mg/mL (low dose) and 7.5 mg/mL (high dose), respectively, and were implanted s.c. at the lower back of the animals. The operations of implanting pumps and inoculating PC-3 cells were carried out simultaneously.

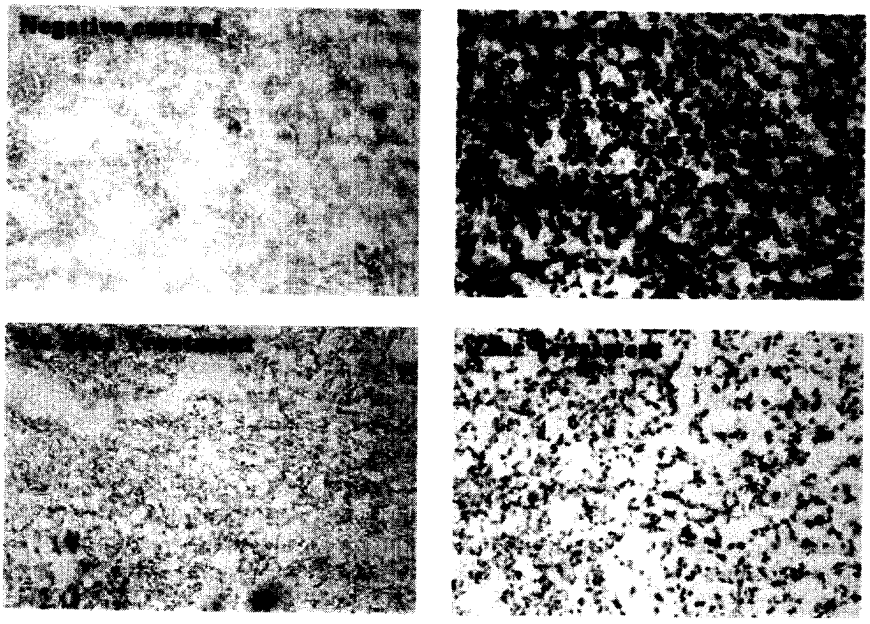

FIGURE 1. Zinc treatment induces apoptosis in tumor tissues: Frozen sections of the tumor tissues (i.m.) were stained for apoptotic DNA fragmentation with an *in situ* cell death detection kit (TUNEL), followed by DAB staining. The positive control was treated with DNase I, and the negative control was assayed without TdT. The results showed that *in vivo* treatment with zinc induced tumor cell apoptosis significantly compared to tumors without zinc treatment.

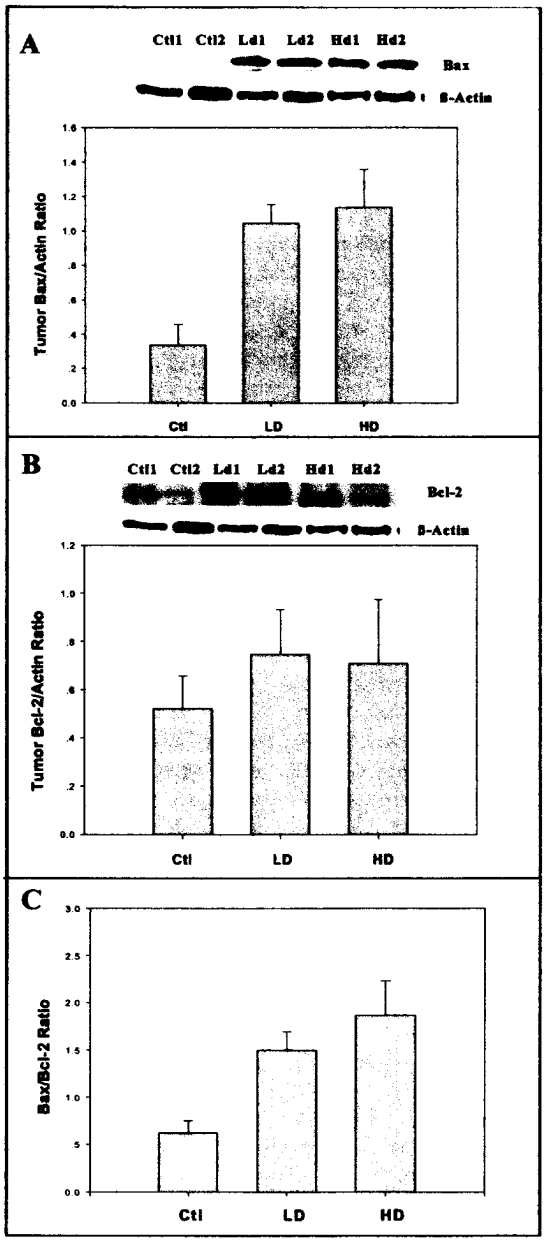

FIGURE 2. The effect of zinc on the ratio of Bax/Bcl-2 levels: **(A)** After 28 days of treatment, the levels of Bax in tumor tissues were detected by Western blot. Significantly increased Bax levels were identified in both zinc-treated groups compared to controls. **(B)** In contrast, Bcl-2 levels increased slightly compared to controls. **(C)** The Bax/Bcl-2 ratio increased significantly in zinc-treated groups, increasing mitochondrial outer membrane permeability and leading to cytochrome *c* release. (Data shown as representatives of the analyzed samples.)

The animal body weights were examined weekly, and their general health conditions were monitored closely. After 28 days of treatment, the mice were sacrificed, and the samples were collected for further studies. Zinc concentrations were determined by atomic absorption spectrophotometer method. Frozen sections of tumor tissues (8 μm) were prepared for TUNEL assay (according to the manufactory protocol) and stained by DAB. The results were examined and recorded by light microscopy.

The levels of Bax and Bcl-2 in the tumor tissues were determined by Western blot analyses with specific antibodies, and the amount of protein loaded for each sample was justified by β actin as an internal control. The citrate level in the tumor tissues was assayed using a previously established fluoroenzymatic method.[3]

The results showed evidence of zinc-induced inhibition of tumor growth in zinc-treated animals by the relatively smaller size and lighter weight of tumors compared with those of the controls. However, there was no significant effect of zinc on animal body weight. The incidence of tumor occurrence was 12/12 (control), 11/12 (low dose), and 9/12 (high dose). A higher zinc accumulation of tumor tissues was observed in zinc treated animals (16.3–16.4 ng/mg protein) than that of controls (12.5 ng/mg protein), leading to about 20–40% increase of citrate production in zinc-treated tumor tissues. In order to investigate the inhibitory effect of zinc on PC-3 cell–induced tumors, frozen sections of tumor tissues were examined by a TUNEL assay (FIG. 1). The results showed that extensive cell apoptosis was observed in zinc treated tumor tissues compared to the controls, in which only a few apoptotic cells were detected. Most recently, we have identified that zinc induces PC-3 cell apoptosis through regulating the mitochondrial outer membrane permeability-related proteins, Bax/Bcl-2 (data not shown) in accordance with previous findings.[4,5] Thus, to further ascertain the mechanism of zinc induced apoptosis *in vivo*, Bax and Bcl-2 levels in the tumor tissues were determined by Western blot (FIG. 2). The results showed that Bax levels significantly increased in zinc-treated tumor tissues compared with those of the controls (about threefold), and that Bcl-2 levels only increased slightly. The ratio of Bax/Bcl-2 in zinc-treated groups was significantly elevated compared to that of the controls.

Our study demonstrates that *in vivo* treatment of zinc increases zinc accumulation and citrate production in PC-3 cell–induced tumor tissues and inhibits tumor growth. The inhibitory effect of zinc appears to result from zinc-induced apoptosis by regulation of mitochondrial membrane permeability-related Bax/Bcl-2 proteins.

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The Involvement of Bax in Zinc-Induced Mitochondrial Apoptosis in Prostate Cells

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ABSTRACT

In selective prostate cells, such as PC-3 cells, the accumulation of zinc under physiological conditions (??) induces mitochondrial apopotogenesis. This is due to a direct action of cytosolic zinc on mitochondria that causes the release of cyt c followed by the caspase cascade that leads to apoptosis. We now provide further information concerning the mechanism of zinc induction of mitochondrial apoptogenesis. Zinc treatment of PC-3 cells or their mitochondria results in an increase in the mitochondrial insertion and polymerization of Bax indicative of pore formation. In addition, exposure of the cells to zinc increases the cellular level of Bax and the Bax/Bcl-2 ratio; which suggests an increase in Bax expression and biosynthesis. These effects are also evident in xenograft PC-3 tumor cells when the host animal is treated with zinc. The cell specificity of the zinc-induced apoptogenesis is dependent upon the responsiveness of the mitochondria to the zinc induction of Bax pore-forming activity.

KEY WORDS: zinc, bax, bcl-2, apoptosis, mitochondria, prostate
ABBREVIATIONS: cyt c, cyt c;

INTRODUCTION

The prostate gland of humans and other animals has the unique ability of accumulating high levels of zinc which account for tissue levels that are 3-10 times higher than found in other soft tissues (Costello 1998). This function resides in the highly specialized secretory epithelial cells, which we characterize as zinc-accumulating cells. The accumulation of high levels of mobile reactive zinc is necessary for the inhibition of citrate oxidation that results in the production and secretion of citrate, which is a major function of the prostate gland. This relationship is in contrast to most other mammalian cells that possess protective mechanisms to prevent against the cellular accumulation of toxic high zinc levels.

A consequence of the accumulation of zinc is the inhibitory effect on growth of these prostate cells that results from its induction of mitochondrial apoptogenesis (Franklin et al, 1995; Liang et al, 1999; Feng et al, 2000). These results from a direct effect of zinc on the mitochondrial release of cyt c followed by caspase activation and cascading apoptotic events (Feng et al, 2000, 2002). However, the mechanism by which zinc induces the release of cyt c had not been addressed or resolved. Recent studies that we now report reveal that zinc treatment of these prostate cells facilitates Bax pore-forming activity and regulates the cellular level of Bax.

Most other reports demonstrate that zinc inhibits apoptosis in mammalian cells (Truong-Tran et al, 2000). Many of those studies employed extremely high levels of zinc (mM range) that would never occur *in situ* even under severe pathological conditions. However some studies have demonstrated that zinc induces apoptosis in selective cells (Provinciali et al, 1995; Hamatake, 2000; Iitaka et al, 2001; Haase et al, 2001; Jiang, 2001). These variable effects of zinc are due to a number of factors; such as the cell type, the concentration of zinc, and other conditions employed in the studies. We now provide evidence that the cell-specific apoptogenic effect of zinc is dependent upon the responsiveness of the mitochondria to zinc facilitation of Bax-associated pore formation.

RESULTS

Direct effect of zinc on mitochondrial resident Bax: We previously demonstrated that exposure of isolated prostate mitochondria to zinc results in the release of cyt c (Feng et al 2002). To further study the molecular mechanism of zinc on mitochondrial apoptogenesis in prostate cells, the direct effect of zinc on

Bax level was determined in isolated mitochondria prepared from PC-3 cells. The mitochondria were exposed to medium supplemented with or without 15 μM zinc for 15 minutes; after which the mitochondria were subjected to alkali treatment to remove loosely-associated Bax (Eskes et al, 2000) and assayed for Bax by Western blot (Fig 1). Zinc treatment resulted in a 400% increase in Bax 23 kDa monomer; an 80% increase in Bax 46 kDa dimer; and a 200% increase in Bax 69 kDa trimer. The results indicate that zinc facilitates the conversion of loosely membrane-associated Bax (which is removed by alkali treatment) to tightly-inserted Bax. In addition, zinc treatment also facilitates the initiation of Bax polymerization, which is indicative of a pore-forming association. As is the case with the release of cyt c (shown in Fig. 8), these results demonstrate that zinc exhibits a direct effect on the mitochondria that does not require the interaction or involvement of additional cytosolic factors. The results also demonstrate that Bax is a resident protein in the prostate mitochondria; i.e. it exists in mitochondria of cells in the absence of an apoptotic signal. In many other mammalian cells, the appearance of Bax association with mitochondria occurs via translocation from cytosol after the cells are exposed to an apoptotic-inducing signal; and in the normal cell condition, Bax is not found as a resident mitochondrial protein (?????).

Effect of zinc treatment of PC-3 cells on mitochondrial Bax: We then determined if the effect of zinc on Bax observed in the isolated mitochondrial preparations also occurred in PC-3 cells that were exposed to zinc-supplemented medium. PC-3 cells were exposed to medium supplemented with 15 μM zinc for 180 minutes vs. no zinc treatment. These are conditions that result in cyt c release and pursuant apoptotic events (Feng et al, 2002). The mitochondria were isolated and the Bax levels determined by Western blot. Fig. 2 reveals that zinc treatment resulted in a 50% increase in the mitochondrial level of 23 kDa monomeric Bax and an increase of 100% of 69 kDa trimerized Bax. Consequently exposure of PC-3 cells to zinc facilitated the translocation and insertion of Bax to the mitochondria as well as the dimerization of Bax. No difference was detected in 46 kDa Bax. In repeated studies we observed that the increase in 23 and 69 kDa was consistently evident; whereas an effect on 46 kDa was variable. This is likely due to the dimer form being a transient step in the polymerization process.

We also determined if zinc treatment of PC-3 cells that overexpress Bax results in amplified polymerized Bax (Fig. 3). The results show the expected increase (~120%) in the Bax monomer level of the PC3+bax cells. In addition the level of Bax trimer was also increased over the control cells by ~60%. Therfore, a significant proportion of the increased expression and synthesis of monomeric Bax must have been translocated to the mitochondria and polymerized to the trimer form.

Effect of zinc treatment on the cellular level and expression of Bax: Because of the apparent increase in mitochondrial Bax, we determined the possibility that zinc might also induce an increase in the cellular level of Bax. PC-3 cells were exposed to zinc-supplemented medium (15 μM zinc) for varying times, and the cellular levels of Bax and Bcl-2 were determined (Fig. 4). Within 3 hrs of exposure to zinc, the cellular level of Bax was increased by ~8-fold. The increase was transient over a 12-hr period. Bcl-2 exhibited a ~1-fold maximal increase. Therefore zinc treatment resulted in a 7-fold increase in the cellular Bax/Bcl-2 ratio. It is generally recognized that maintenance of an appropriate Bax/Bcl-2 balance in cells prevents apoptosis (Thornborrow and Manfreddi, 2001). When this ratio is altered in favor of Bax, cells become apoptotic due to increased translocation of Bax to mitochondria.

To obtain some initial insight into the mechanism associated with the increased cellular level of Bax, we determined the effect of zinc on the level of Bax-mRNA by RT-PCR (Fig 5). Exposure of PC-3 cells to 15 μM zinc resulted in a transient increase in Bax-mRNA at 60 minutes, but not at 30 minutes, this is consistent with the later transient increase in Bax protein. These results are suggestive of a zinc-induced increase in Bax gene expression (?????).

Effect of overexpression of Bcl-2 on zinc-induced apoptosis of PC-3 cells: The results of the experiments described above indicate that zinc induction of mitochondrial apoptogenesis involves a Bax-associated pore-forming process. To corroborate this possibility, one would expect that over-expression of Bcl-2 should attenuate zinc-induced apoptosis. PC-3 cells transfected with Bcl-2 (pCEP4/Bcl-2) or

with vector only (pCEP4) were cultured to near confluence and then exposed to zinc-supplemented medium (15 μM) for 24 hrs. Cells proliferation and apoptotic characteristics were determined along with cellular level of Bcl-2 (Fig. 6). The results reveal that zinc exhibited its apoptotic effect in the control cells (pCEP4). In contrast, Bcl-2 transfected cells exhibited a marked decrease in the rate of apoptosis. The success of the transfection is revealed by the pronounced increase in the cellular level of Bcl-2 in the transfected cells. Collectively, these results are consistent with the proposal that zinc facilitates Bax-associated pore formation that initiates mitochondrial apoptogenesis.

***In vivo* effects of zinc on Bax levels and apoptosis of PC-3 tumors in nude mice:** The studies described above consistently demonstrate the apoptogenic effects of zinc *in vitro* (on the cells and isolated mitochondria). However we were interested in determining if corresponding effects of zinc could be observed in an *in vivo* model. In association with our prostate tumorigenesis project (Feng et al, 2003), we determined the level of Bax and apoptosis in PC-3 induced tumors in nude mice. The effect of zinc treatment of animals on tumor growth was determined. The conditions employed resulted in an increase in the plasma level of zinc in zinc-treated animals compared to control animals and significantly decreased the growth of tumors (Feng et al, 2003). Most relevant to this current study, Fig. 7 shows that the tumor cells from zinc-treated animals exhibited an increase in zinc levels; and also exhibited an approximate 3-fold increase in Bax in contrast to a slight but insignificant increase in the Bcl-2 level, thereby resuling in an approximate 2-fold increase in the Bax/Bcl-2 ratio. The TUNEL assay reveals a marked increase in the apoptotic cell population of the tumors from zinc-treated animals. These *in vivo* results parallel and corroborate the *in vitro* effects of zinc.

Cell specificity of zinc induced mitochondrial apoptogenesis: We previously reported (Feng et al, 2000, 2002) that zinc induction of mitochondrial apoptogenesis is cell specific in that it occurs in zinc-accumulating prostate epithelial cells (as represented by human prostate cell lines LNCaP, BPH, and PC-3, and by freshly isolated normal rat ventral prostate epithelial cells), but not in cells that do not accumulate high zinc levels (squamous carcinoma cell line, HPV and HPR-1 prostate cell lines)???. Fig. 8 shows that, in contrast to PC-3 cells, zinc treatment of HPR-1 does not induce apoptosis and does not induce the release of cyt c from isolated mitochondria. Most importantly, in contrast to PC-3 cells, the mitochondria isolated from HPR-1 cells do not exhibit any detectable polymerization of Bax (69 KD and 46 KD) either in the presence or absence of zinc treatment. Also, the level of tightly bound monomeric Bax (23 KD) is barely detectable in the control and zinc-treated groups of HPR-1 mitochondria. Thus, in HPR-1 cells a correlation exists among a) the absence of a zinc effect on mitochondrial Bax, b) the absence of zinc-induced release of cyt c, and c) the absence of zinc-induced mitochondrial apoptogenesis.

DISCUSSION

The results of this study confirm and extend our previous reports (Feng et al, 2002, 2003) that zinc induces mitochondrial apoptogenesis in select zinc-accumulating prostate epithelial cells. It is now evident that the mechanism involves zinc effects on Bax. Zinc exhibits a direct effect on mitochondria that facilitates the insertion and polymerization of resident Bax, which are activities that are involved in the mitochondrial pore-forming process (Eskes 2000, Grinberg 2002, Kroemer and Reed 2000). This is consistent with the release of cyt c, which occurs in response to zinc treatment. Consequently, the zinc-induced release of cyt c might be due to its facilitation of Bax-associated pore formation. Jiang et al (2001) similarly reported that zinc directly induced the release of cyt c from neuronal mitochondria. They reported that the release of cyt c followed the rapid zinc-induction of mitochondrial swelling, which apparently was associated with opening of PTP. Presently we have no direct information that this action might be applicable to the zinc effect on prostate mitochondria. However, under the conditions of our study, zinc does not uncouple mitochondrial respiration (unpublished information); which would be an expected consequence of mitochondrial swelling. Some evidence does exists for the possibility that apoptotic signaling in cells results in the translocation of Bax to the mitochondria followed by Bax

interaction with ANT of the PTP (Desager and Martinou, 2000; Halestrap et al, 2002; Bewlzacq et al, 2002). Zinc might induce Bax homo-oligomerization and hetero-oligomerization with Bak that results in new pore-formation; or zinc might facilitate the co-localization of Bax with ANT or VDAC that leads to pore-opening. In this regard it is important to recognize that the structure of Bax does not reveal the presence of any potential zinc-binding sites (Suzuki et al, 2000, PDP accession no.1F16); whereas, ANT dimer does contain potential zinc binding sites (Halestrap 2002). Consequently we are now conducting critical time-course studies to establish the inter-relationship of the events in response to zinc.

In addition to its direct effect on isolated prostate mitochondria, in vitro and in vivo studies demonstrated that zinc treatment also results in the increase in the cellular level of Bax and in the Bax/Bcl-2 ratio; which are conditions generally considered to facilitate apoptogenesis in mammalian cells (Thornborrow and Manfreddi, 2001). It would seem that this effect of zinc is in concert with its direct effect on mitochondria in that additional Bax becomes available for facilitation of mitochondrial apoptogenesis (Fig 3). Although the cause of the increase in cellular Bax levels is yet to be established, the increase in Bax mRNA (Fig 5) indicates a likely stimulatory effect of zinc on Bax gene expression. Bax gene expression is up-regulated by a variety of apoptosis-inducing factors (Antonnson 2001). The delayed transient increase in Bax-mRNA is indicative of an intermediate-late gene response to zinc, rather than a direct immediate-early response. In parallel studies (unpublished studies) we observe that zinc induction of metallothionein (MT) gene expression (increase in MT-RNA) in PC-3 cells is evident within fifteen minutes. Unlike the MT genes that contain metal response elements (MREs), a search of the promoter region of the Bax gene reveals the absence of consensus sequences of MRE. Therefore a direct interaction with Metal-binding Transcription Factor for activation of an MRE is not an available option for an immediate-early Bax gene regulation by zinc. However, several response elements for transcription factors (TFs) reportedly activated by zinc are present in the Bax promotor. For example, Egr-1 also known as Zif/268, or NGFI-1 is an immediate-early gene that is induced by zinc (Park and Koh, 1999). A variant of the hypoxia-inducible gene (HIF-1) is also induced by zinc (Chun et al, 2001? 2002). Binding sites for both of these TFs are present in the Bax promoter. Interestingly, recent evidence has established that artificial TFs containing 3 or 5 Zifs, based on the DNA binding domain of Egr-1/Zif268, selectively regulated Bax gene expression (Falke, Fisher and Juliano, 2003). Thus a mechanism involving zinc induction of these TFs with subsequent induction of Bax expression does exist and would be consistent with the time course of zinc-induced Bax expression.

The apoptogenic effect of zinc in mammalian cells is divergent; being an inducer of apoptosis in some cells and an inhibitor of apoptosis in other cells. Reasons for the divergent cell specific effects of zinc have not been identified although the variable experimental conditions employed in different studies are likely contributing factors. The present report and our previous studies (Liang et al, 1999; Feng et al,2000; 2002) demonstrate that the mitochondrial apoptogenic effect of physiological levels of zinc is specific for zinc-accumulating prostate cells. The specificity is dependent upon the ability of cells to accumulate intracellular levels of mobile reactive zinc sufficient to induce apoptogenesis. In addition, we previously showed that the cell specificity is also dependent upon the ability of the mitochondria to release cyt c in response to zinc exposure (Feng et al, 2002). It is now evident that the ability of the mitochondria to respond to zinc involves its facilitation of the Bax response. We now have a model to establish the mechanism for the cell specific mitochondrial Bax response to zinc.

MATERIALS and METHODS

Cell Lines and Cell Cultures: PC-3 cells, a human malignant prostate cell line obtained from American Type Culture Collection (ATCC, Rockville, MD), were maintained in RPMI-1640 medium with glutamine, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. HPR-1 cells

(provided by Dr. Ck Choo, University of Hong Kong, China), an epithelial cell line, was derived from normal human prostate. HPR-1 cells were cultured in serum-free keratinocyte medium supplemented with EGF (2.5 μg/500ml), bovine pituitary extracts (25 mg/500ml) (GIBCO, Invitrogen Corp., Carlsbad, CA) and 1% penicillin/streptomycin as described previously (Feng et al 2002). The passages of the cells were within the range of 10-28.

Zinc treatment of the cells: Generally, at 24 h before zinc treatment, the medium was changed to RPMI-1640 without FBS for PC-3 cells and growth factors- free keratinocyte medium for HPR-1 cells to minimize any effect of undefined components that might influence the effect of zinc on the cells. Then the cells were treated with zinc for a period of times as indicated in the Results section. The cells were then used for isolation of mitochondria and/or preparation of total cell protein. The isolated mitochondria were subject for alkaline extraction.

Mitochondrial Preparation and zinc treatment of isolated mitochondria: PC-3 cells were collected by centrifugation at 250 x g for 5 min at 4°C. The cells were washed with ice-cold PBS twice and re-suspended in 5 volumes of mitochondrial isolations buffer (MIB) composed of 220 mM mannitol, 68 mM sucrose, 10mM KCl, 1 mM EDTA, 1mM EGTA, 10 mM HEPES, 0.1% bovine serum albumin (BSA), with added fresh protease inhibitors (pepstatin A, 5μg/ml; leupeptin, 10μg/ml; aprotinin, 2μg/ml), pH 7.4. The cells were homogenized gently on ice with a glass homogenizer and followed by a centrifugation at 800 x g for 10 min. The resulting supernatant fluid was centrifuged at 10,000 x g for 5 min at 4°C. The pellet (mitochondria) was resuspended in MRB buffer composed of 200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, 10 mM HEPES, 0.1% BSA, pH 7.4, and kept on ice. The protein concentration of mitochondrial suspension was measured by the method of Bradford (Bradford et al 1976). Aliquotes of the mitochondrial suspension (100 μg of protein) were then adjusted to the final reaction (100 μl/reaction) by adding 1μl of each following compound, ATP (100 mM), dATP (100 mM) and $MgCl_2$ (50 mM) and exposed to zinc (10 ng/μl) at 30 °C for 15 min. At the end of the incubation period, the mitochondria were separated from the reaction by rapid centrifugation at 10,000 x g for 5 min and the mitochondrial pellets were the subject for alkaline extraction.

Alkali extraction of mitochondria and Detection of Bax insertion and levels of Bax, Bcl-2 by Western Blot: For alkaline extraction (Eskes et al 2000), the mitochondrial pellets were re-suspended (1 mg protein/ml) in freshly prepared 0.1 M Na_2CO_3 (pH 11.5) and incubated for 20 min on ice. The mitochondrial membranes were then collected by centrifugation (360,000 x g for 20 min at 4°C). The levels of Bax and Bcl-2 in the samples prepared from the isolated mitochondria directly treated with/without zinc; the mitochondria isolated from the cells, which were treated with/without zinc; and the cells treated with/without zinc, were detected by Western blot analyses as described previously (Feng et al 2000). Western blot assays were conducted with specific anti-Bax (Santa Cruz Biotechnology, Inc., CA) and anti-Bcl-2 (BD Biosciences Pharmingen, CA), and the amount of protein loaded for each sample was justified by the signals of β actin as an internal control.

TUNEL assay for detection of apoptosis: Frozen sections (8 μm) of tumor tissues were prepared for TUNEL assay using In Situ Cell Death Detection Kit, POD (Roche, Germany). The

experiments were conducted according to the manufactory protocol and the samples were stained by DAB. The results were examined and recorded by computer connected light microscopy.

Transiant transfection of PC-3 cells in cultures: The expression constructs and corresponding vectors of human Bax/pEGFP-C3 and Bcl-2/pCEP4 were provided by Dr. R.J. Youle (NINDS, NIH) (Wolter KG et al 1997). In preparation for transfection, PC-3 cells were plated at a density of $2x10^5$ cells/well in six-well tissue-culture plates (COSTAR, Corning Incorporated, Corning, NY), 24h later, cells were transiently transfected using the TransIT-Prostate Transfection Kit (Mirus, Madison, WI) for Bax and PolyFact Transfection Reagent (QIAGEN, Valencia, CA) for Bcl-2. The transfection was conducted using 2-3 μg of plasmid DNA per well at the conditions as manufacturer recommended. At 18-24 hrs after the transfection, the cells were either collected for Western blot analysis or continued for further experiments.

Prostatic tumorigenicity in nude mice: Male nude mice (4-6 weeks) were housed in a pathogen-free environment under controlled light and humidity. Tumors were established by inoculation of PC-3 cells (5-10x 10^6/ml) in 10% Metrigel and using 0.1 ml for each injection s.c. at both flanks of the animals. Zinc treatment was given by ALZET osmotic pumps (Durect Corp., Cupertino, CA), with a releasing rate of 0.25 μl/h for 28 days. The pumps were filled with PBS (control) and zinc sulfate 10 mg/ml; and the pumps were implanted at the lower back of the animals. The operations of implanting pumps and inoculating of PC-3 cells were carried out simultaneously.

Semi-quantitative RT-PCR of Bax mRNA: PC-3 cells treated with/or without zinc for variable time period were collected and total RNA of the cells was extracted using TRIzol™ reagent (GIBCO, Invotrogen) according to the manufacturer's protocol. Two μg of total RNA was used for reverse transcription (RT) reaction (Roche, Indianapolis, IN) at final volume 100μl, and then 10 μl of RT reaction was used as template in 25μl of PCR reaction (Taq polymerase: Invitrogen Life Technologies) to detect Bax gene expression (human Bax primers: TCAAGACCACTCTTCCCCACACCCC; GCCCACCAGCTCTGAGCAGATCAT, DNA fragment length 677bp) and human GAPDH (primers: CCACCCATGGCAAATTCCATGGCA; TCTAGACGGCAGGTCAGGTCCACC, DNA fragment length 598bp) was used as an internal control. The PCR program protocol is: 95°C for 3 min, then followed by 94°C 45sec, 53°C 45 sec, 72°C 1 min 30 sec for 35 cycles and finished by a final step at 72°C for 7 min. PCR results were analyzed by electrophoresis. The density of bands was scanned using AlphaEase Image system. The ratio of Bax vs. corresponding GAPDH represents the levels of Bax mRNA.

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TITLES AND LEGENDS TO FIGURES

Fig. 1. Direct effect of zinc on Bax integration into mitochondrial membrane. Isolated mitochondria (100 μg) from PC-3 cells were incubated with or without zinc (10 ng/μg protein) for 15 min at 30°C and followed by alkaline extraction. The reactions were analyzed by Western blot for Bax and the results were expressed by arbitrary units, which were relative optical densities corrected by corresponding results of β-actin (same as in the following figures).

Fig. 2. Zinc effect on mitochondrial integration of Bax was detected in zinc treated PC-3 cells. PC-3 cells were treated with or without zinc (1000 ng/ml) for 3 hrs and mitochondria were then isolated and extracted by alkaline. The changes of Bax were determined by Western blot analyses.

Fig. 3. Effect of zinc on mitochondrial integration of Bax in PC-3 cells with over-expression of Bax. PC-3 cells were transiently transfected with Bax gene constructs. Forty eight hrs after the transfection the cells were treated with or without zinc (1000ng/ml) for 3 hrs; then mitochondria were isolated and treated by alkaline. The changes of mitochondrial Bax were determined by Western blot analyses.

Fig. 4. Effect of zinc on the ratio of cellular Bax/Bcl-2 levels. PC-3 cells were exposed to zinc (1000ng/ml) for 3, 6 and 12 hrs period of time; cells without zinc treatment were used as a control. The cellular levels of Bax and Bcl-2 were determined by Western blot analyses, and the ratio of Bax/Bcl-2 was expressed by the ratio of arbitrary units corresponding to the densities of Bax and Bcl-2, respectively.

Fig. 5. Effect of zinc on Bax mRNA levels in PC-3 cells determined by RT-PCR. PC-3 cells were cultured in 6-well plates and treated with or without zinc (1000ng/ml) for various times as indicated. Each sample was analyzed by semi-quantitative RT-PCR, and the levels of Bax were corrected by GAPDH used as an internal control for PCR. The changes of Bax mRNA levels in zinc treated groups were expressed by the ratio of Bax_{zinc}/Bax_{ctl}.

Fig.6. Effect of over-expressed Bcl-2 on zinc-induced apoptosis of PC-3 cells. PC-3 cells were transiently transfected with pCEP4-Bcl-2 constructs or pCEP4 vector alone. The levels of Bcl-2 were determined by Western blot analysis (lower panel). The transfected cells were exposed to zinc (1000 ng/ml) for 24 hrs, and the apoptotic effect of zinc was detected by light microscopy (upper panel).

Fig.7. *In vivo* effect of zinc on Bax /Bcl-2 ratio and apoptosis of PC-3 derived tumors in nude mice. The levels of Bax and Bcl-2 of tumor tissues from the control and zinc treated animals (n=6) were detected by Western blot analyses (left of lower panel); and the ratio of Bax/Bcl-2 is shown in the right of lower panel. Zinc-induced cell apoptosis was identified in frozen tumor tissue sections (8μm) by TUNNEL assay (shown as brown stained apoptotic bodies in the right of upper panel).

Fig.8. The cell specificity of zinc-induced apoptosis in PC-3 and HPR-1 cells. A. The cells were treated with and without zinc (1000 ng/ml) for 24 hrs, and the cell morphologic changes were observed by light microscopy. B. The isolated mitochondria were treated with zinc (0.01 and 0.02 μM) for various times as indicated; zinc-induced cytochrome c release from isolated

mitochondria was determined by Western blot. C. Zinc effect of Bax integration into mitochondria membrane was determined in zinc-treated isolated mitochondria by Western blot.

Fig.1

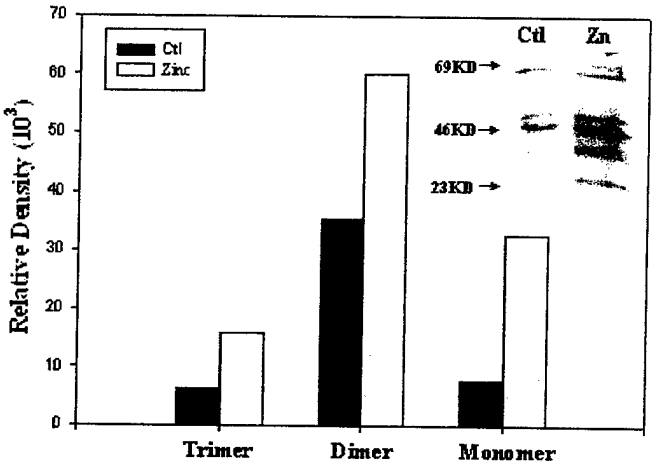
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Fig.2

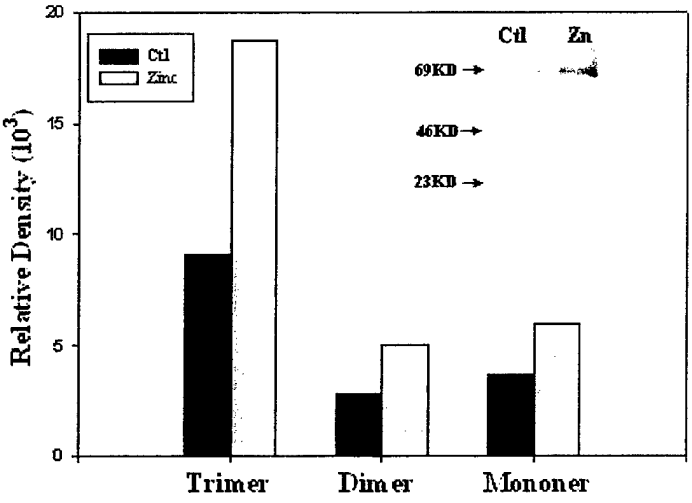
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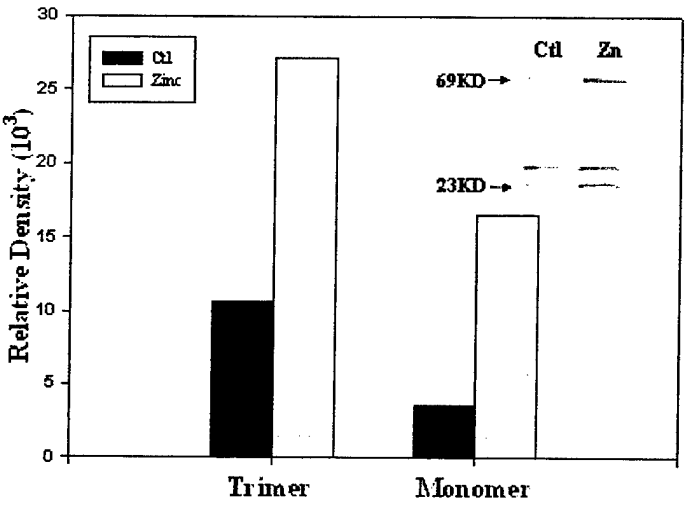
Fig.3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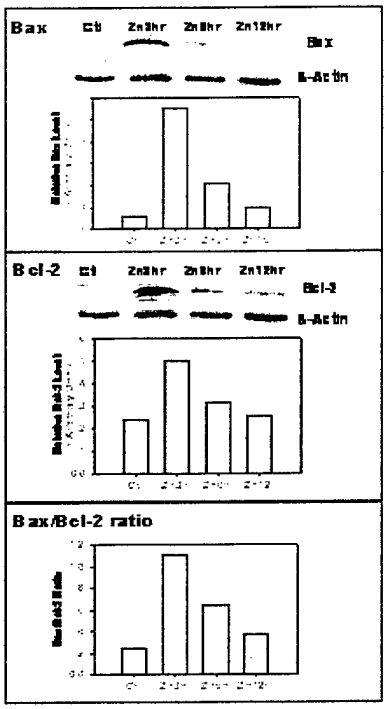
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Fig.5

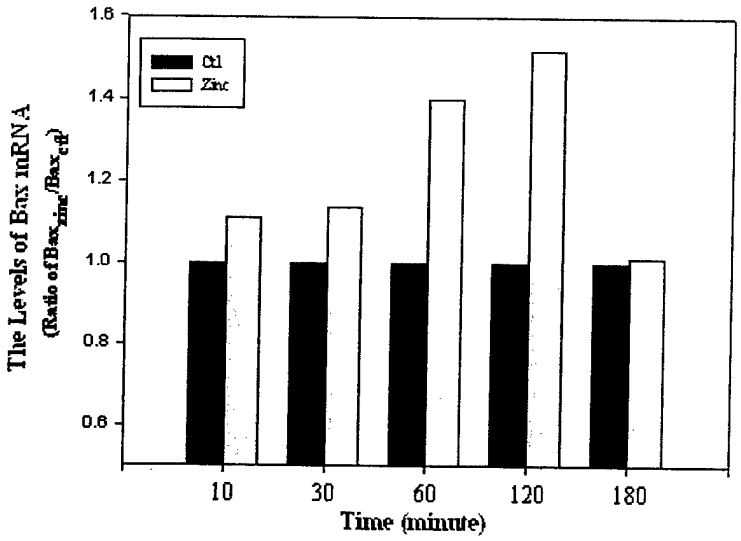
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Fig.6

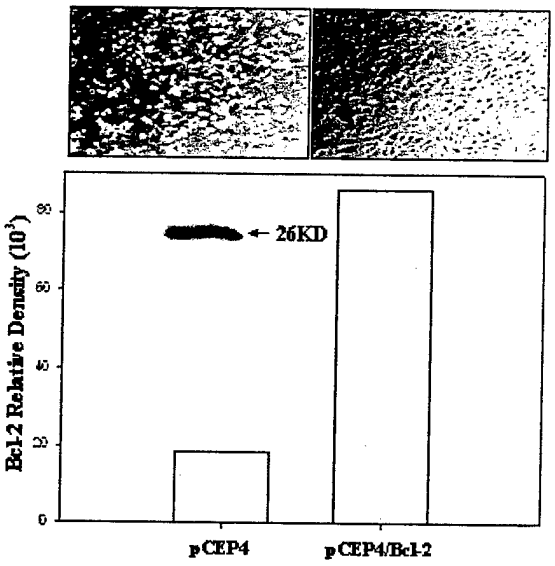

Fig.7

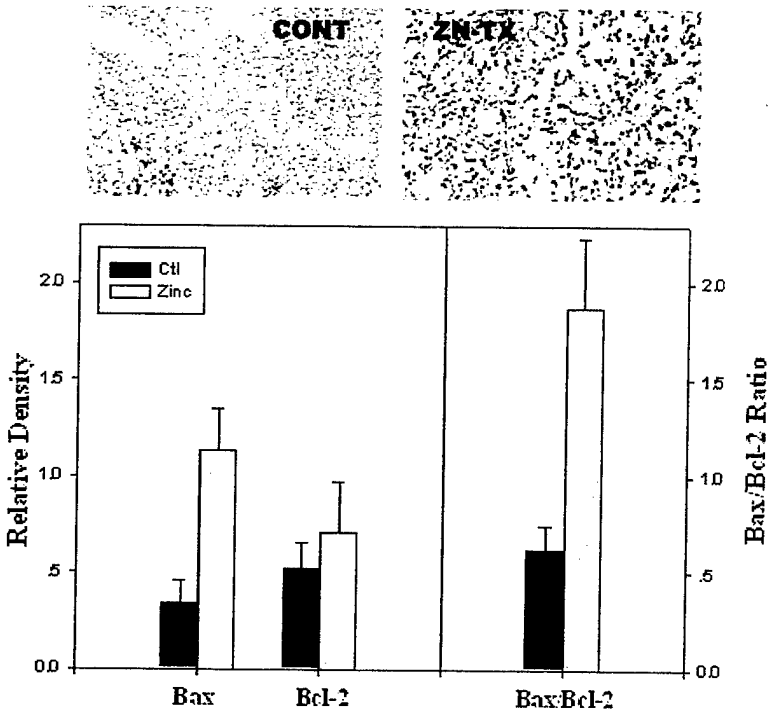
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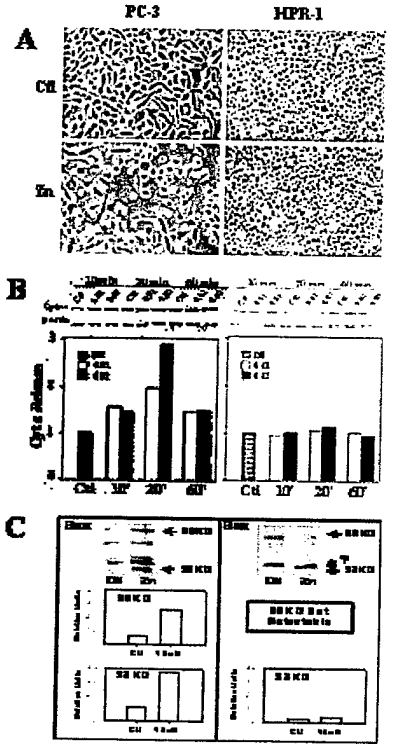
Fig.8



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Role of zinc in the pathogenesis and treatment of prostate cancer: critical issues to resolve

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The most consistent and persistent biochemical characteristic of prostate cancer (PCa) is the marked decrease in zinc and citrate levels in the malignant cells. This relationship provides compelling evidence that the lost ability of the malignant cells to accumulate zinc is an important factor in the development and progression of prostate malignancy. In addition, this relationship provides a rational basis for the concept that restoration of high zinc levels in malignant cells could be efficacious in the treatment and prevention of PCa. Epidemiological studies regarding dietary zinc effects on PCa have been conflicting and confusing. The purpose of this presentation is to present a current state of information regarding zinc relationships in the pathogenesis and treatment of PCa. We also hope to bring more attention to the medical and research community of the critical need for concerted clinical and basic research regarding zinc and PCa.
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Keywords: zinc; prostate cancer; zinc transporters; prostate epithelial cells; apoptosis; citrate oxidation

Introduction

In previous publications,[1–3] we reviewed and detailed the compelling evidence and basis for the concept that zinc is involved in the pathogenesis of prostate cancer (PCa); and that zinc could be efficacious in the prevention and treatment of PCa. Since those earlier reviews, significant additional information has become available regarding the zinc relationship in prostate. We can best frame the discussion by addressing the following questions: 'Is altered zinc metabolism an important factor in the pathogenesis of prostate cancer?' Can zinc be efficacious against the development and progression of prostate cancer? Our intention in this presentation is to provide a comprehensive update of current information, to address contemporary issues and conflicting reports, and to expand the awareness of the scientific and clinical community regarding the implications of zinc in prostate cancer. For details of earlier related studies, which will be minimized in this presentation, we refer the reader to our previous reviews.[1–5]

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The major function of zinc in the normal human prostate

For the following presentation, the human prostate gland must be anatomically defined. In this discussion, we will consider the prostate to be comprised of the peripheral zone (about 70%), the central zone (about 25%), and the transition zone (about 5%). In regard to zinc relationships and the development of malignancy, the peripheral zone is the major component. The central gland normally contains much lower zinc levels than in peripheral zone. Therefore, the normal glandular secretory epithelial cells of the peripheral zone, but not the central gland, are zinc-accumulating cells. In benign prostate hyperplasia (glandular BPH) the zinc levels in the central gland are dramatically increased and often exceed the concentration that exists in normal peripheral zone. The transition zone is believed to be the origin of BPH. The following presentation focuses on the zinc relationships to prostate cancer; and, unless otherwise defined, relates to the peripheral zone.

A major function of the peripheral zone glandular epithelium is the production and secretion of extraordi-

narily high levels of citrate (Table 1). The peripheral zone glandular secretory epithelium also accumulates high levels of zinc; three to 10-fold higher than other soft tissues. The uniqueness of this function becomes more apparent when one recognizes that mammalian cells generally possess mechanisms that prevent the accumulation of high zinc levels; particularly mobile reactive zinc that can have toxic effects. Why then does this function exist in these highly specialized secretory epithelial cells? The answer is that a high level of zinc in mitochondria is essential to inhibit m-aconitase activity.[6] This prevents the oxidation of citrate, which accumulates and is secreted (Figure 1). In other mammalian cells, the inhibition of m-aconitase and citrate oxidation is lethal. This emphasizes the uniqueness of the highly specialized prostate secretory epithelial cells.

Table 1 Representative citrate and zinc levels is prostate

	Citrate	Zinc
Normal (mixed tissue)	8000	209
Normal (central zone)	4000	121
Normal (peripheral zone)	13 000	295
BPH	8000–15 000	589
PCa (mixed tissue)	1000–2000	55
PCa (malignant tissue)	500	—
Other soft tissue	150–450	30
Blood plasma	90–110	1
Prostatic fluid	40 000–150 000	590

Citrate values are nmol/g wet weight. Zinc values are μg/g wet weight.

The apoptogenic effect of zinc

In addition to its major function and metabolic effect described above, the accumulation of high zinc levels can have other consequences. One such effect is the inhibition of cell growth, which results from its induction of mitochondrial apoptogenesis.[7–9] This effect has been identified in human prostate cell lines (PC-3, LNCaP, BPH-1) and in normal rat ventral prostate epithelium, all of which are capable of accumulating high zinc levels when exposed to zinc-supplemented medium. In contrast, the human prostate HPr-1 cell line and the squamous carcinoma cell line, which are not zinc-accumulating cells, do not exhibit an apoptogenic response to zinc treatment. In most mammalian cells, zinc reportedly prevents apoptosis. Therefore, the apoptogenic effect of zinc is specific for zinc-accumulating cells. This effect results from a direct action of zinc on the mitochondria, which results in the release of cytochrome *c* that leads to apoptotic cascading events (Figure 2). The cell specificity is dependent upon two factors. (1) the ability of the cells to take up and accumulate zinc, and (2) the ability of the mitochondria to respond to the increased cytosolic level of zinc.

The mechanism of zinc accumulation

Why are these prostate epithelial cells capable of accumulating cellular levels of zinc that are several-fold higher than most other mammalian cells although they share the same interstitial fluid environment? Presently, the mechanisms responsible for the high cellular zinc

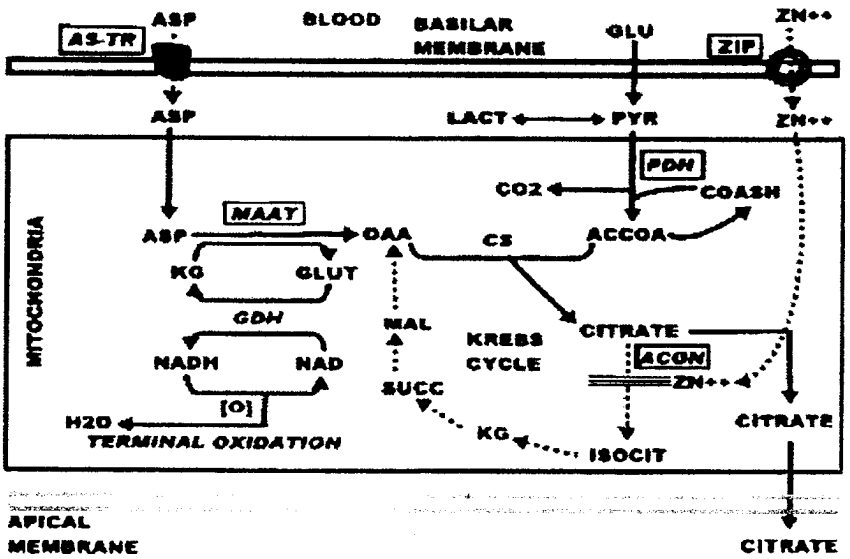

Figure 1 *Role of zinc in the pathway of net citrate production.*

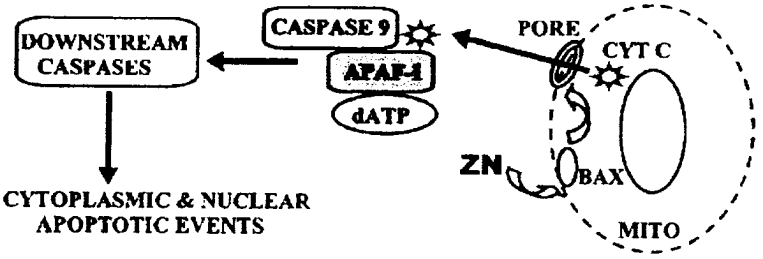

Figure 2 *Mechanism of zinc-induced mitochondrial apoptogenesis in prostate cells.*

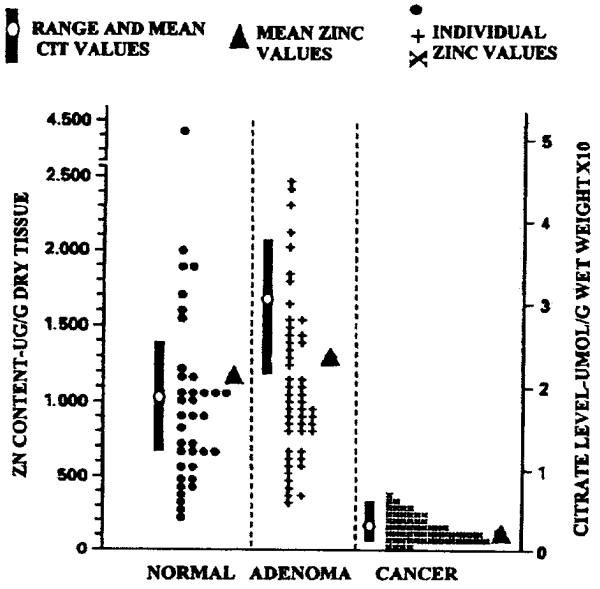

Figure 3 *Composite results of zinc and citrate levels in prostate cancer, benign hyperplasia, and normal individuals. The citrate values were determined by* in situ *magnetic resonance spectroscopy of the prostate.*[14] *The zinc values were determined by energy dispersive X-ray fluorescence of resected prostate tissue.*[13]

level are unknown. Recent studies[10–12] have shown that the zinc transporter, ZIP1, is important in the uptake and accumulation of zinc by prostate cells. Upregulation of ZIP1 in prostate cells increases zinc accumulation, which inhibits cell growth and increases net citrate production. This reveals that a significant component of the accumulated cellular zinc is retained as mobile reactive zinc. Correspondingly, downregulation of ZIP1 decreases zinc accumulation in prostate cells. The net accumulation of zinc would also be dependent upon the export of zinc out of the cell. Although, a number of zinc export transporters have been identified, their functional relationship in cellular zinc accumulation has not been established.

Zinc levels in prostate cancer

As represented in Table 1 many studies (reviewed and cited in Costello and Franklin[1]) have consistently demonstrated a marked decrease in prostate tissue zinc levels in PCa *vs* normal prostate or BPH samples. Clearly, the uniquely high zinc levels that characterize the normal glandular epithelium of the peripheral zone are greatly reduced (generally 70–80% lower) in the malignant tissue. Figure 3 compares the zinc values from the study of Zaichick *et al*[13] with the citrate values from the study of Liney *et al*.[14] Most importantly, there exists no individual case where the malignant tissue has retained either the high zinc level or citrate level of the normal peripheral zone. This consistent relationship exists despite the variations in patient populations, in stage of prostate cancer, in tissue sampling, in assay procedures, and other variables. Also important is the observation that the decrease in zinc and in citrate occurs early in malignancy.[15,16] This is confirmed by the *in situ* magnetic resonance spectroscopy identification of decreased citrate in malignant loci in the peripheral zone of cancer subjects (for review, see Costello *et al*[4,5]). Decreased citrate is now the most specific and consistent marker for malignant loci in the prostate gland. This decrease occurs by the time that any histopathological evidence of malignancy is apparent. Since zinc is the cause of citrate production, its decrease precedes the decrease in citrate, and this metabolic transformation is initiated prior to the overt appearance of malignancy; that is, in a premalignant stage (Figure 4).

The impact of decreased zinc level in malignancy

We have identified two important effects of decreased zinc levels; a metabolic effect, and a growth effect. The metabolic effect results from the release of zinc inhibition of m-aconitase, which then permits the oxidation of citrate via the Krebs cycle (Figure 1). This has a major effect on the bioenergetics of the cell. The inhibition of citrate oxidation at the aconitase step eliminates the coupled energy (ATP) production that normally occurs from Krebs cycle oxidation. Under such conditions, the aerobic oxidation of glucose results in the production 14 ATP/glucose, as contrasted with 38 ATP/glucose that results when citrate oxidation exists (Figure 1). Thus, the malignant cells become energy-efficient cells in contrast to the energy-inefficient, specialized citrate-producing secretory epithelial cell. This provides the additional energy production that is required for the malignant cell to perform its potential malignant activities. The growth effect results from the elimination of the apoptogenic influence of zinc, which, in combination with the metabolic effect, permits the proliferation of the

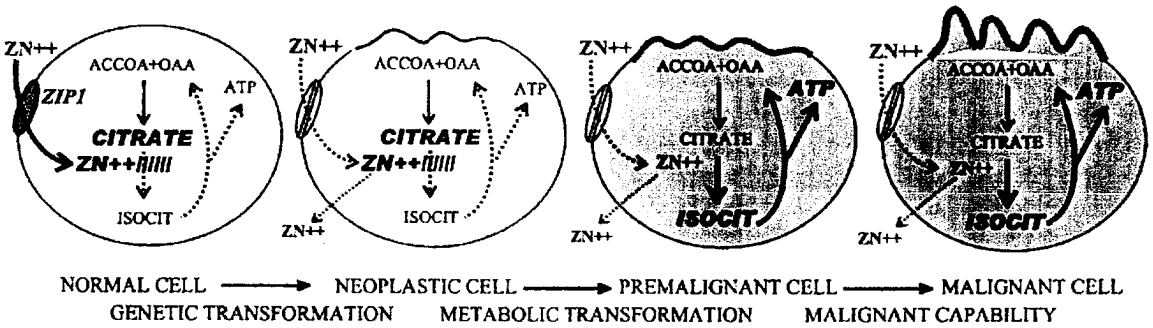

Figure 4 *Concept of pathogenesis of prostate malignancy. The normal cell possesses the zinc-accumulating apparatus (ZIP1). The high zinc levels in mitochondria inhibit m-aconitase resulting in the inability to oxidize citrate and the accumulation of citrate. The neoplastic cell has lost the ability to accumulate zinc. As the cellular zinc levels are decreased in the premalignant cell, citrate oxidation occurs with the concurrent increased production of ATP. The malignant cell is now metabolically and energetically capable of proceeding with its malignant process. Another effect (not shown in this figure) of the decrease in zinc is the removal of its apoptogenic effect, which then permits the proliferation of the malignant cells.*

malignant cells. These events will not occur if the malignant cell retains a high zinc accumulation, which explains why zinc-accumulating, citrate-producing malignant cells are not found in prostate cancer. However, this concept does not suggest or imply that the lost ability of peripheral zone epithelial cells to accumulate zinc is the cause of the development of malignant cells. A genetic mutation to a neoplastic cell with malignant potential is essential. Once such a neoplastic cell develops, the zinc-associated metabolic transformation is essential for the manifestation of its malignant activities. Figure 4 is a representation of this concept of the pathogenesis of prostate malignancy.

What is the cause of the decrease in zinc accumulation?

The elucidation of the cause of the lost ability of the malignant cells to accumulate zinc will be critical in understanding the pathogenesis of PCa. A possible reason is the involvement of ZIP1 since it is now known to be important in the uptake and accumulation of zinc in prostate cells.[10,11] We recently showed that the downregulation of ZIP1 is accompanied by a decrease in the uptake of zinc by prostate cells. Most importantly, Rishi *et al*[12] reported that ZIP1 and ZIP2 expression was downregulated in malignant loci of the peripheral zone as compared to its expression in adjacent normal tissue. Moreover, expression of both transporters is lower in normal peripheral zone from black males as compared to white males, which coincides with the race-associated higher incidence of PCa in African-Americans. Correspondingly, we have identified (unpublished information) that the level of ZIP1 protein (immunocytochemistry with ZIP1 antibody) is markedly decreased in the malignant loci of peripheral zone as compared with high ZIP1 level in the adjacent normal glandular epithelium. Consequently, evidence is evolving that downregulation of expression of the ZIP transporters is a cause of decreased zinc uptake and accumulation in malignant cells; and this altered gene expression might be a critical factor in the development of PCa. This leads to the future possibility that restoration of ZIP transporter gene expression could prevent prostate malignancy.

In situ zinc effects in peripheral zone *vs* effects in prostate cells *in vitro*: a conundrum?

The relationships described herein evolved from the combination of *in vitro* studies and *in situ* studies. The issue always arises as to the applicability of *in vitro* studies to the 'true' *in situ* conditions. This is especially relevant to the zinc relationships. While it is clearly established that the malignant cells *in situ* in PCa (ie 'true' malignant cells) have lost the ability to accumulate zinc, malignant cell lines such as LNCaP and PC-3 have retained the ability to accumulate zinc under *in vitro* conditions. The ability of these cells to accumulate zinc is not evidence that the 'true' malignant cells have not lost the ability to accumulate zinc. It is evidence that the 'true' malignant cells retained the potential ability to accumulate zinc; but, under the *in situ* conditions, either the zinc-accumulating apparatus is suppressed or not expressed, and/or zinc export mechanisms prevent the cellular retention of imported zinc. Nor does this difference demonstrate that the cell lines are inappropriate models for representation of 'true' malignant cells. It is essential to recognize these circumstances in order to employ appropriate experimental conditions and the limitations imposed by these circumstances.

Another important issue relates to the apoptogenic effect of zinc. We identified this effect in *in vitro* studies with prostate cell lines and freshly isolated rat ventral prostate cells. Then the question is raised, 'Why don't the normal secretory epithelial cells of the peripheral zone (ie 'true' normal epithelial cells), which accumulate very high zinc levels, exhibit a high rate of apoptogenesis and die?' If they do not die, is it likely that zinc does not have an apoptogenic effect on the 'true' normal secretory epithelial cells? The answer does not reside in a conclusion that the zinc effect on apoptogenesis that occurs *in vitro* is not an effect of zinc *in situ*. In fact, the apoptogenic effect does occur *in vivo* as shown in the

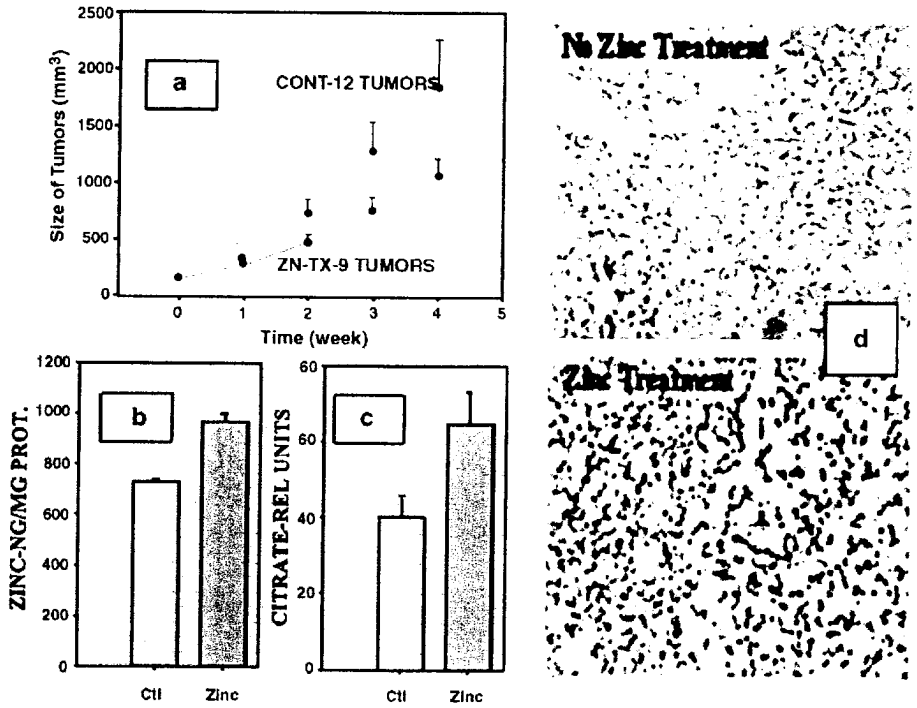

Figure 5 *Effect of zinc treatment of mice on PC-3 tumor growth.* (**a**) *Tumor growth.* (**b**) *Tumor zinc level.* (**c**) *Tumor citrate level.* (**d**) *Tumor cell apoptosis.*

tumorigenic study described below (Figure 5). The explanation resides in the *in situ* conditions of the 'true' secretory epithelial cells, which must modulate the apoptogenic effect of zinc. Indeed, this should be expected since these are highly specialized cell that evolved for the function of zinc accumulation; and, therefore must have adapted mechanisms to protect the cells from the toxic influences of zinc.

These currently unresolved relationships are not conundrums. There exist appropriate answers. They signal the necessity to elucidate the factors and conditions that represent the natural environment and *in situ* operation of the malignant cells and the normal epithelial cells from which they are derived.

The case for zinc treatment in prostate cancer

It is apparent that the malignant prostate cells virtually never exist as zinc-accumulating citrate-producing cells. The metabolic transformation to neoplastic cells that have lost the ability to accumulate zinc and are citrate-oxidizing cells is essential for the manifestation of their malignant activities. Therefore, it is plausible to apply this relationship to the treatment of PCa, and perhaps to the prevention of clinically manifested PCa. An approach is to create conditions that will enhance the uptake and accumulation of zinc in the malignant cell (for treatment) or in the premalignant cell (for prevention). The simplest regimen is to increase the circulating level of zinc to enhance the uptake of zinc. In this regard it is important to note that zinc bioavailability (plasma zinc and tissue levels) generally decline with aging.[17] There is some evidence, albeit still inconclusive, that decreased plasma zinc level might be associated with PCa;[18–20] but no reports that increased plasma zinc is associated with PCa. Nevertheless, a significant increase in circulating zinc, especially in a form that is available for cellular uptake, could be efficacious. The obvious mode for increasing the circulatory level of zinc would be by oral ingestion of supplemental zinc; but some problems exist with this approach as described below.

Effective cellular uptake of zinc does not occur by simple diffusion. The lost ability to extract zinc from circulation is due to the downregulation of the uptake transport process (eg ZIP transporters). Therefore, another approach is to restore or enhance the uptake transport process of the malignant cells. In this regard, we have shown that testosterone and prolactin can increase zinc uptake in prostate cells;[21] and these hormones upregulate ZIP1 expression.[10] Therefore, the application of an appropriate hormonal regimen to enhance zinc uptake in the malignant cells could be successful. A combination of increased circulating zinc and hormonal (such as prolactin) stimulation of zinc uptake could provide the most efficacious approach.

To test this concept, we recently conducted preliminary zinc treatment studies in an experimentally induced human prostate tumorigenic model; that is PC-3 cell-induced tumors in nude mice.[22] Subcutaneous-implanted minipumps containing zinc chloride solution provided a sustained release of zinc. This route was selected to provide a more direct and better-controlled source of zinc to circulation than oral ingestion. The success of this treatment is revealed by a significant increase in the plasma zinc level. Over a five-week

period, the zinc-treated animals exhibited a 50% reduction in tumor growth (Figure 5). Coinciding with this was an increase in the zinc and citrate levels of the tumors from the zinc-treated group, which reveals an increase in mobile reactive zinc. Consequently, zinc treatment metabolically altered the tumor cells to citrate-producing cells. Also apparent is a marked increase in apoptosis in the tumors from zinc-treated animals; thereby, demonstrating that the apoptogenic effect occurs *in vivo* as well as *in vitro*. Consequently, in this experimental model, all these effects are compatible with the concept described above. The critical question is whether or not this will translate into corresponding effects of zinc treatment in PCa in humans.

Epidemiological studies: what have they revealed?

Several case–control epidemiological studies that involved the efficacy of dietary zinc against PCa have been reported;[23–29] (for reviews see Thomas,[30] Platz and Helzlsouer,[31] and Kolonel[32]). Most reports[26–29] indicated that dietary zinc had no apparent beneficial or harmful effect on PCa risk. Kristal *et al*[24] reported a significant reduction in the risk of PCa by zinc supplement; and Key *et al*[25] also concluded that zinc might be beneficial against PCa. In contrast, Leitzmann *et al*[23] reported an increase in the risk of PCa by excessive zinc supplement. Obviously, no unanimity or consensus regarding the effects of dietary zinc on PCa can be derived from these epidemiological studies. Indeed, the dominant conclusion of most of these studies is that further studies are essential and warranted.

It is unfortunate and disturbing that the conclusion of Leitzmann *et al*[23] has been echoed in public announcements. The Washington Post (July 1, 2003, Reuters) announced, 'Men who take too much zinc may be raising their prostate cancer risk, U.S. researchers said yesterday.' HealthDay News (July 2, 2003) recounts, 'Men who overdose on zinc supplements more than double their risk of prostate cancer, a government study finds.' Excluded from the report of Leitzmann *et al* is any reference to or discussion of any previously reported epidemiologic studies; none of which are corroborative of their conclusions. The omission of such highly relevant information and lack of explanation for the apparent diverse observations lead the reader to an erroneous supposition that their report is the sole and incontrovertible study. In our view, this conclusion is unfounded, and the message now in the public domain is not warranted. Indeed, we hope that our presentation will provide a balanced assessment of the current state of the available scientific/medical information regarding this important issue.

One must ask why no consistency or consensus has been derived from these epidemiological studies. In some instances the database, the patient selection, confounding factors, and the application of statistical analyses are problematic. However, the major explanation resides within the complexity of interacting multiple dietary factors that affect the intestinal absorption and assimilation of zinc (for reviews see Lonnerdal,[33] Krebs,[34] and King *et al*[35]). For example the level of phytate, iron, calcium, and numerous other ingested nutrients can inhibit the absorption of zinc. Also, the absorption of zinc varies with the ligand form of zinc. An important factor that has not been considered in these epidemiological studies is the effect of zinc concentration on the efficiency of its absorption. The intestinal uptake of zinc provides a homeostatic mechanism that regulates the systemic level of zinc. A low-intestinal zinc level upregulates zinc absorption, and an excessive intestinal zinc level downregulates zinc absorption. The supposition of most of the epidemiological studies has been that the amount of ingested zinc is approximated by the amount of zinc that is absorbed and assimilated. However, when all these interacting factors are considered, this supposition becomes untenable. Any direct effect of zinc on normal or malignant prostate cells is dependent on the circulating level of zinc and its uptake by the cells; not on the intestinal level of zinc. The most important information is the effect of the ingested level of zinc on the systemic level of zinc; and that information is lacking in these epidemiological studies. It is interesting to note that several reports have demonstrated that plasma zinc levels were either normal or decreased, but not increased, in prostate cancer subjects;[18–20] which further argues against a deleterious effect of elevated systemic zinc. It should be apparent that such epidemiological studies have not and likely will not establish the role and effectiveness of zinc in PCa.

Summary

Prostate malignancy involves the metabolic transformation of normal zinc-accumulating citrate-producing cells to citrate-oxidizing malignant cells that have lost the ability to accumulate zinc. Malignant cells in PCa virtually never contain high zinc levels. The lost ability to accumulate zinc is an essential metabolic transformation that is required for the manifestation of the malignant activities of the neoplastic malignant prostate cell.

Downregulation of ZIP (zinc uptake) transporter gene expression might be responsible for the lost ability to accumulate zinc; and, therefore, could be a significant genetic factor in PCa.

Overwhelming evidence supports the concept that restoration of high zinc levels in the malignant or premalignant cells could be efficacious against the development of overt malignancy or the progression of malignancy.

Epidemiological studies regarding the effectiveness of dietary zinc in the risk of PCa have been conflicting and inconclusive.

Well-controlled studies of the efficacy of zinc alone and in combination with other supplements are required, along with basic research studies to establish the mechanisms of regulation of zinc and its effects on normal and malignant prostate.

Acknowledgements

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Direct Effect of Zinc on Mitochondrial Apoptogenesis in Prostate Cells

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BACKGROUND. Prostate epithelial cells uniquely accumulate significantly higher levels of zinc than other mammalian cells. We previously showed that the accumulation of high intracellular zinc levels in specific prostate cells results in the induction of apoptosis and the inhibition of cell growth. The apoptotic effect is due to zinc induction of mitochondrial apoptogenesis. We now report additional studies that corroborate this effect of zinc and provide insight into the mechanism of this unique effect.

METHODS. The effect of exposure to physiological levels of zinc on apoptosis was determined for three human prostate cell lines (PC-3, BPH, and HPR-1). Zinc-induced apoptosis was identified by DNA fragmentation. The direct effect of zinc on isolated mitochondrial preparations from each cell line was determined. The mitochondrial release of cytochrome c was determined by Western blot.

RESULTS. Exposure to zinc induced apoptosis in PC-3 and BPH cells but not in HPR-1 cells. The zinc accumulation in PC-3 (4.3 ± 0.3) and BPH (2.8 ± 0.4) was higher than that in HPR-1 cells (1.8 ± 0.1). The apoptotic effect of zinc on PC-3 cells could be observed as early as 4–6 hr of zinc treatment, and this effect was not reversible. The exposure of isolated mitochondria from PC-3 and BPH cells to zinc resulted in the release of cytochrome c; but zinc had no effect on mitochondria from HPR-1 cells.

CONCLUSIONS. Exposure to zinc induces apoptosis in PC-3 and BPH cells, which accumulate high intracellular levels of zinc, but not in HPR-1 cells, which do not accumulate high levels of zinc. Once initiated, the induction of apoptosis is not reversed by the removal of zinc, i.e., it is an irreversible process. The apoptogenic effect is due to a direct effect of zinc on mitochondria that results in the release of cytochrome c. The cell specificity of zinc induction of apoptogenesis is dependent on the ability of the cells to accumulate high levels of intracellular zinc and on the ability of the mitochondria to respond to the direct effect of zinc.
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KEY WORDS: **zinc; prostate; mitochondria; apoptosis; PC-3; BPH; HPR-1**

INTRODUCTION

Normal human prostate secretory epithelial cells accumulate the highest zinc levels of any cells in the body. In prostate cancer (PCa), the malignant prostate cells have lost this ability; compelling evidence now exists for the implication of zinc in the pathogenesis and progression of prostate malignancy [1–3]. Consequently, zinc plays a critical role in the normal function and pathology of the prostate gland. For detailed reviews of these relationships, see Costello and Franklin [3,4].

An important question is, "What are the consequences and role of the accumulation of high zinc levels in the prostate secretory epithelial cells that

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possess the unique capability of transporting and accumulating high levels of zinc (i.e., zinc-accumulating prostate cells)?" We recently demonstrated that zinc accumulation in prostate cells results in increased apoptosis [5,6] and that this effect was manifested by the zinc induction of mitochondrial apoptogenesis [6]. Exposure of these cells to zinc rapidly induces the translocation of cytochrome c from mitochondria into cytosol, which triggers caspase activation that leads to cascading cytosolic and nuclear apoptotic events. In contrast, exposure of cells to zinc that are incapable of high zinc accumulation does not result in apoptogenesis. This present report confirms and extends our earlier reports and addresses two important issues relating to the mechanism of zinc-induced mitochondrial apoptogenesis: (1) whether or not the effect of zinc is due to a direct effect of zinc on the mitochondria, and (2) if the cell-specific effect of zinc is due to the level of cellular zinc and/or in the capability of mitochondria to respond to accumulated zinc.

MATERIALS AND METHODS

Cell Lines and Cultures

The present studies were conducted with PC-3 cells, a human malignant prostate cell line obtained from ATCC as we previously described [5]; BPH cells (kindly provided by Dr. S. Haywood, University of California, San Francisco), an epithelial cell line that was derived from human benign prostatic hyperplasia (BPH) tissue; and HPR-1 cells (kindly provided by Dr. C. K. Choo, University of Hong Kong, Hong Kong, China), an epithelial cell line that was derived from normal human prostate tissue. PC-3 cells and BPH cells were cultured in RPMI-1640 medium with 10% (PC-3) and 5% (BPH) fetal bovine serum and 1% penicillin/streptomycin. HPR-1 cells were cultured in serum-free keratinocyte medium with EGF (2.5 μg/500 ml) and bovine pituitary extracts (25 mg/500 ml) (Gibco BRL, Life Technologies, Bethesda, MD) and 1% penicillin/streptomycin.

Mitochondrial Preparation and Detection of Cytochrome c Release

The cells were collected by centrifugation at 250*g* for 5 min at 4°C. The cells were washed with ice-cold PBS twice and resuspended in 5 volumes of mitochondrial isolation buffer (MIB) composed of 220 mM mannitol, 68 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES, 0.1% bovine serum albumin (BSA), with added fresh 1 mM DTT and protease inhibitors (pepstatin A, 5 μg/ml; leupeptin, 10μg/ml; aprotinin, 2μg/ml), pH 7.4. The cells were homogenized gently on the ice with a glass homogenizer and followed by a centrifugation at 800 × g for 10 min. The resulting supernatant fluid was centrifuged at 10,000*g* for 5 min at 4°C. The pellet (mitochondria) was resuspended in MRB buffer composed of 200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, 10 mM HEPES, 0.1% BSA, pH 7.4, and kept on ice. Aliquots of the mitochondrial suspension (200 μg of protein/40-μl reaction) were exposed to zinc for various time periods at 30°C under conditions described in the Results section. At the conclusion of the incubation period, the mitochondria were separated from the reaction by rapid centrifugation at 10,000*g* for 5 min. The supernatant fluid was assayed for cytochrome c by Western blot. The protein concentration of the mitochondrial preparations was determined by the method of Bradford [7]. Western blot assays were performed with specific anti-cytochrome c and β-actin antibodies (BD Transduction Laboratories, San Diego, CA) under the conditions recommended by the manufacturer.

Detection of Cell Apoptosis

The extraction of DNA and detection of DNA fragmentation were performed as previously described [5]. The morphology of the cells treated with or without zinc in six-well culture plate for designated time periods and the characteristics of apoptotic cells were observed under an inverted microscope (Nikon, Eclipse TE200) and photographed.

Determination of Cellular Zinc

Prostatic cells were grown in 75 cm^2 flasks until 90% confluence of the culture. The cells were treated with or without zinc (1,000 ng/ml) in fresh serum-free medium for 3 hr. Before harvest, the cells were washed once with 1 × PBS and then washed twice after the collection to remove extracellular zinc. The cells were resuspended in sucrose buffer (250 mM sucrose, 20 mM HEPES, pH 7.4) and homogenized on ice. The nuclei and cell membranes were separated by centrifugation at 800*g* for 10 min. The supernatants were then centrifuged at 10,000*g* for 5 min, and these supernatants were used as cytosol samples. The protein concentrations of the samples were measured by Bradford method [7]. Thirty microliters of each sample (200 μg of protein) were placed in a 96-well plate and mixed well with 60 μl of TSQ buffer, which was composed of 1.9 g of sodium acetate, 2.9 g of sodium barbital, 1.5 mg of TSQ (Molecular Probes, Eugene, OR) dissolved in 100 μl of warmed ethanol, then double distilled H_2O added to 100 ml, pH 10. The fluorescence of zinc labeled by TSQ was detected by using a Fluoroskan Ascent, Labsystems, Microplate

Reader (Life Sciences International Company, USA) with excitation of 355 and emission of 485 [8,9].

In this study, all experiments were repeated three or more times to establish the consistency and reproducibility of the results. The data presented are representative of the results of the repeated experiments.

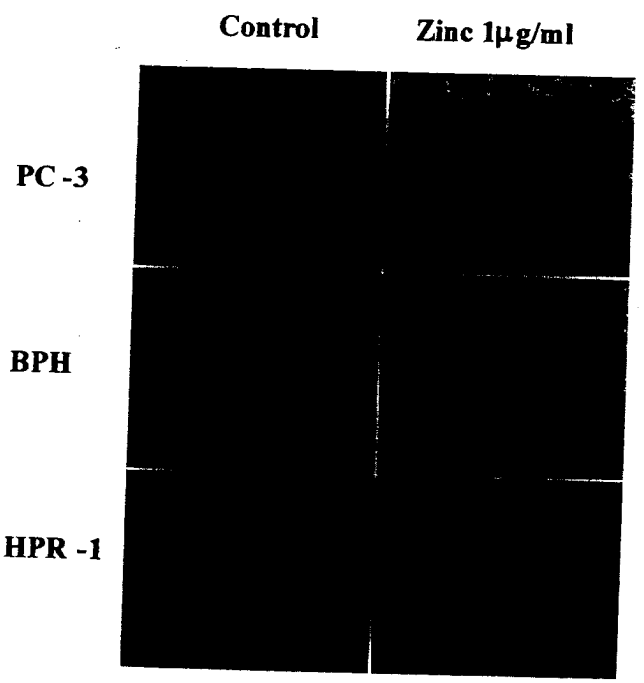
RESULTS

Zinc Induces Apoptosis in Human Prostatic Malignant (PC-3) and Benign Hyperplasia (BPH) Cells, but Not in HPR-1 Cells Derived From Normal Prostatic Epithelial Cells

We previously demonstrated that exposure of LNCaP and PC-3 cells to physiological levels of zinc results in increased apoptosis and decreased cell growth while having no effect on HPV-1 cells, which are derived from human normal prostatic epithelial cells [5,6]. Correspondingly, LNCaP and PC-3 cells will accumulate high zinc levels; whereas HPV-1 cells do not accumulate high zinc levels (unpublished information). To extend this important relationship, the effect of zinc on the induction of apoptosis was studied in BPH cells, in HPR-1 cells that, like HPV-1 cells, purport to be representative of normal human prostate secretory epithelial cells, and in PC-3 cells for comparison of the effects. Figure 1 reveals that zinc exposure (1 μg/ml for 24 hr) of PC-3 and BPH cells resulted in a marked decrease in the cell density while having no effect on HPR-1 cells. Correspondingly, cell apoptotic morphologic features were observed in both PC-3 and BPH cells treated with zinc but no similar effect was observed in HPR-1 cells. The apoptotic changes observed in PC-3 and BPH cells included the appearance of cell membrane "blebbing," nuclear chromosome condensation, and eventually the formation of apoptotic bodies. Apoptosis was further confirmed by genomic DNA fragmentation (Fig. 2). Multiple 180-bp DNA fragments, resulting from internucleosomal cleavage, were clearly visible in PC-3 and BPH cells treated with 0.25, 0.50, and 1.0 μg/ml zinc for 24 and 48 hr. To determine that these effects were possibly associated with a cellular accumulation of zinc, the level of increased zinc accumulation in the zinc-treated cells vs. the control cells was determined to be 4.3-fold for PC-3 cells, 2.7-fold for BPH cells, and only 1.8-fold for HPR-1 cells. The values for PC-3 and HPR-1 cells are consistent with the cellular increases as measured by atomic absorption (unpublished information). Thus, zinc induction of apoptosis is a major factor in the inhibitory effect of zinc on prostate cell growth as we reported earlier [5].

A time-course study of zinc-induced apoptosis was further conducted in PC-3 cells (Fig. 3A). The results show that zinc induction of apoptosis in PC-3 cells is time dependent. Apoptotic cells appeared as early as 4 hr after zinc treatment, and apoptosis was clearly evident after 6 hr of zinc treatment. Between 6 and 24 hr after the start of zinc treatment, the increase in apoptotic cells was associated with significant decreased cell density.

In another study (Fig. 3B), we wanted to determine whether zinc-induced apoptogenesis could be reversed by removal of the zinc. PC-3 cells were recovered for 24 hr in normal growth medium after 2,



Zinc Accumulation in Cytosol

	Control	Zinc 1μg/ml	Zinc/Control
PC-3	4.2 ± 0.4	18.1 ± 2.4	4.3 ± 0.3
BPH	7.5 ± 1.1	20.5 ± 0.9	2.8 ± 0.4
HPR-1	9.0 ± 1.3	14.1 ± 1.2	1.8 ± 0.1

Fig. 1. Accumulation of zinc, which induces apoptosis in human prostatic malignant PC-3 and BPH cells but not in human HPR-1 cells derived from normal prostate epithelial cell. The cells were cultured in six well/plate at a density of approximately 4×10^5 cells/well in the growth medium. Twenty-four hours before the zinc treatment, growth medium was replaced by serum and/or growth hormone-free medium. Zinc (1,000 ng/ml) was then added for another 24 hr. Triplicate cultures were used for each treatment. Cell morphologic features were observed and photographed under a microscope (Nikon, Eclipse, TE200). Table: The cellular accumulation of zinc was measured by TSQ as described in the Materials and Methods.

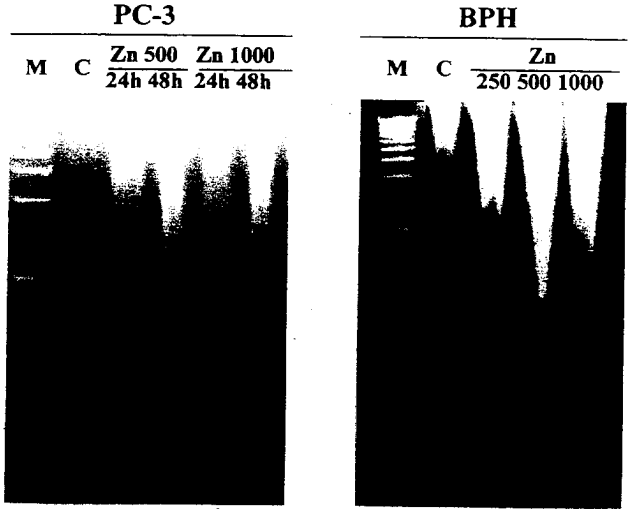


Fig. 2. Effect of zinc on DNA fragmentation in PC-3 and BPH cells. Shown is a representative electrophoretic fragmentation pattern of DNA isolated from PC-3 and BPH cells treated with or without zinc (250, 500, and 1,000 ng/ml) for 24 (PC-3 only) and 48 hr. Medium and the zinc treatment were replaced every 24 hr.

4, 6, and 24 hr of zinc (1 μg/ml) treatment. It is clear that as early as 4–6 hr of exposure of the cells to zinc irreversibly induced apoptosis and subsequent decreased cell density. The longer time of zinc treatment, the more extensive apoptosis appeared. This recovery study of zinc treatment showed that the inhibitory effect of zinc on PC-3 cell growth is not reversible.

Zinc Induction of Cytochrome c Release in Isolated Mitochondrial Preparations

We reported previously that the induction of apoptosis by zinc in PC-3 cells involved the release of cytochrome c from mitochondria into cytosol, which then triggered caspase activation leading to downstream apoptotic events [6]. However, a key unresolved issue was whether the accumulated cellular zinc acted directly on the mitochondria to release cytochrome c, or if the accumulated zinc triggered some other cellular event that was required to initiate the release of cytochrome c. To address this issue, the direct effect of zinc on isolated mitochondrial preparations was determined (Fig. 4). Figure 4A reveals that exposure of isolated mitochondria from PC-3 cells to either 38 μM or 76 μM zinc (equivalent to 0.01 and 0.02 μmol zinc/mg mitochondrial protein, respectively) significantly increased (approximately 50%) the release of cytochrome c within 10 min of exposure. Further increases (90% and 190% for 38 μM and 76 μM zinc, respectively) of cytochrome c release were evident after 20 min of exposure to zinc. Even after the prolonged period of 60 min of exposure during which the endogenous release of cytochrome c is increased, zinc treatment resulted in the additional (50%) release of cytochrome c. The effects of zinc on cytochrome c release from BPH mitochondria were essentially the same as the effects obtained for PC-3 mitochondria (Fig. 4B). Thus, it is clearly evident that zinc-induction of the release of cytochrome c is due to a direct and rapid effect of zinc on mitochondria. These results extend our earlier report, which showed that zinc induces mitochondrial apoptogenesis in intact PC-3 cells by means of a stimulatory effect on the release of cytochrome c [6].

It is extremely important to note that (Fig. 4C), in sharp contrast to PC-3 and BPH, neither 38 μM nor 76 μM zinc had an effect on the release of cytochrome c from isolated mitochondria from the HPR-1 cells. This finding is consistent with the absence of zinc induction of apoptosis in these cells under conditions that induce apoptosis in PC-3 and BPH cells (Fig. 1). The results reveal that after zinc treatment the lower cytosol accumulation of zinc by HPR-1 cells as shown in Figure 1 was not the only reason for the absence of an apoptotic effect of zinc. The endogenous level of cytosol zinc is higher in HPR-1 cells than in BPH and PC-3 cells; yet these HPR-1 cells do not exhibit apoptosis (Fig. 1). It is now evident that the mitochondria in these cells, unlike PC-3 and BPH mitochondria, are not responsive to the direct effects of zinc on the release of cytochrome c.

The results presented in Figure 4 indicated that PC-3 and BPH mitochondrial exposure to zinc for 20 min was optimal for the release of cytochrome c. It was then important to conduct a dose-response study to determine the minimal concentration and the range of zinc that could result in the release of cytochrome c (Fig. 5). Studies with BPH mitochondria reveal that a dose-response effect of 20-min exposure to zinc on the release of cytochrome c is evident over the range of 0.002–0.040 μmol zinc/mg protein (equivalent to 7.6–152 μM zinc). Over this range, the cytochrome c released increased from 44% (at 0.002 μmol/mg protein) to approximately 300% (at 0.04 μmol/mg protein) when compared with control mitochondria.

DISCUSSION

These studies extend our previous observations that zinc accumulation in prostate cells induces mitochondrial apoptogenesis. Most importantly, it is now evident that the induction of mitochondrial apoptogenesis is due to a direct effect of zinc on the mitochondria. Heretofore, it was thought that the cellular accumulation of zinc could have inducible effects that were prior to and required for the manifestation of the

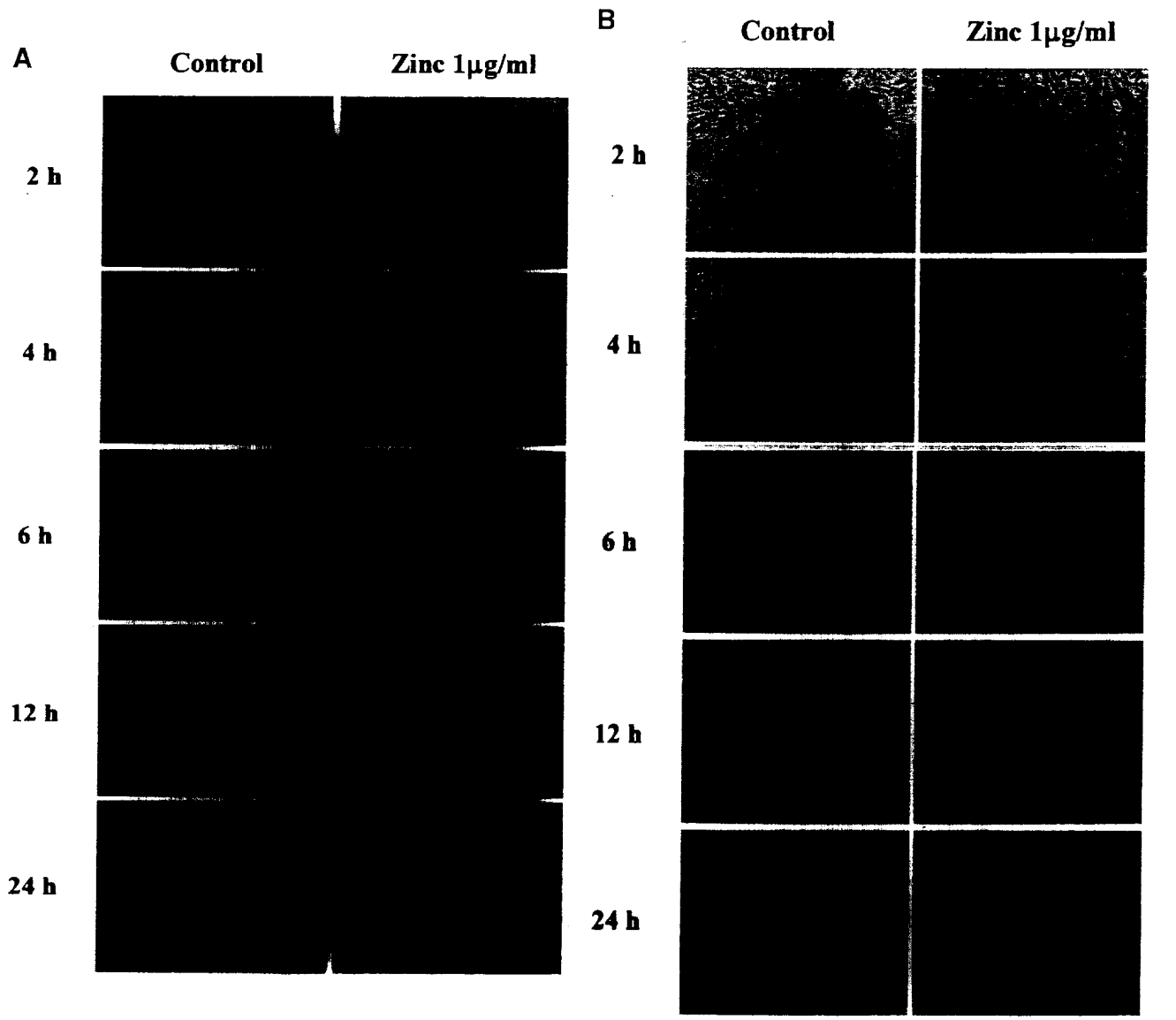
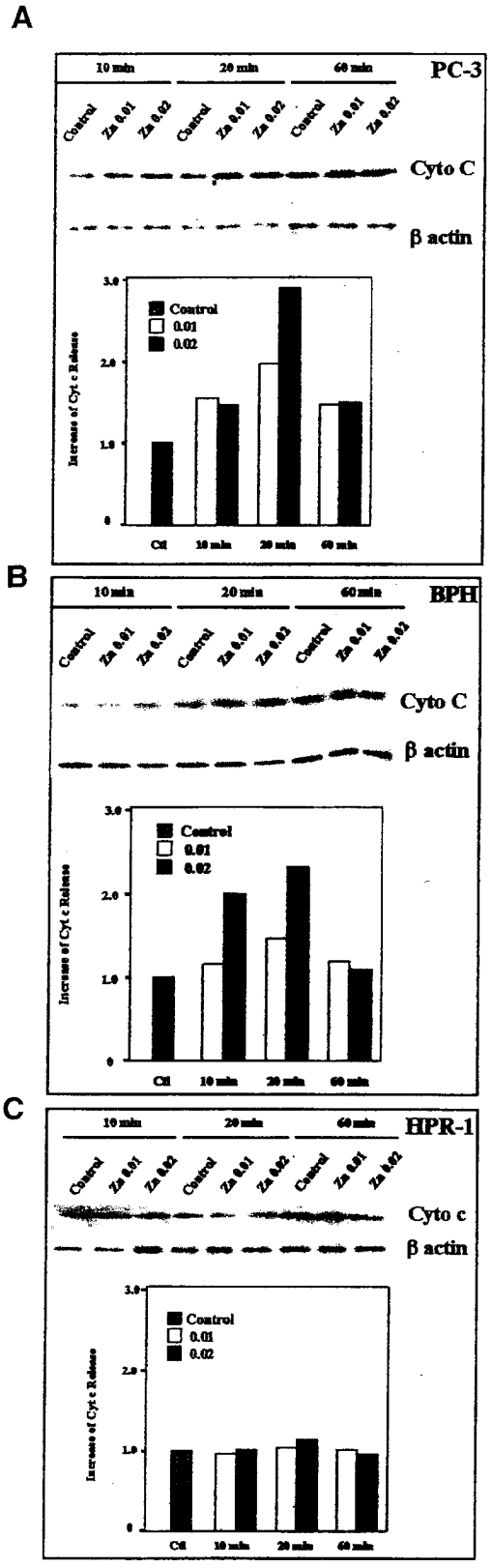


Fig. 3. The inhibitory effect of zinc on PC-3 cell growth is time-dependent (**A**) and is not reversible (**B**). PC-3 cells were treated with (right panels) or without (left panels, control) zinc (1,000 ng/ml) for 2–24 hr, as indicated. After each treatment, zinc medium was removed and cells were washed once with serum medium and followed by another 24-hr culture in fresh growth medium (B). The cell morphologic changes were observed and recorded by photograph.

mitochondrial response. That zinc induces the release of cytochrome c from isolated prostate mitochondria eliminates any required interaction of zinc on non-mitochondrial conditions or components before its mitochondrial effect. The next critical issue to investigate is the mechanism by which zinc alters mitochondrial function that leads to the release of cytochrome c and the onset of apoptogenesis.

It is extremely important to note the difference between the PC-3 and BPH cells vs. the HPR-1 cells. PC-3 cells, BPH cells, and, as previously reported [10], LNCaP cells accumulate high zinc levels when exposed to physiological levels of extracellular zinc. Correspondingly, the accumulation of zinc induces mitochondrial apoptogenesis in these cells. In addition, freshly prepared rat ventral prostate epithelial cells also undergo mitochondrial apoptogenesis when exposed to zinc [6]. Clearly, in zinc-accumulating prostate cells, exposure to zinc results in apoptogenesis. In contrast, the exposure of PZ-HPV cells (purported to be derived from normal peripheral zone) to zinc does not result in the cellular accumulation of high zinc levels and, therefore, does not undergo zinc-induced apoptosis [6]. Similarly, HPR-1 cells do not accumulate high zinc levels and do not exhibit zinc-induced apoptogenesis. Evidently the



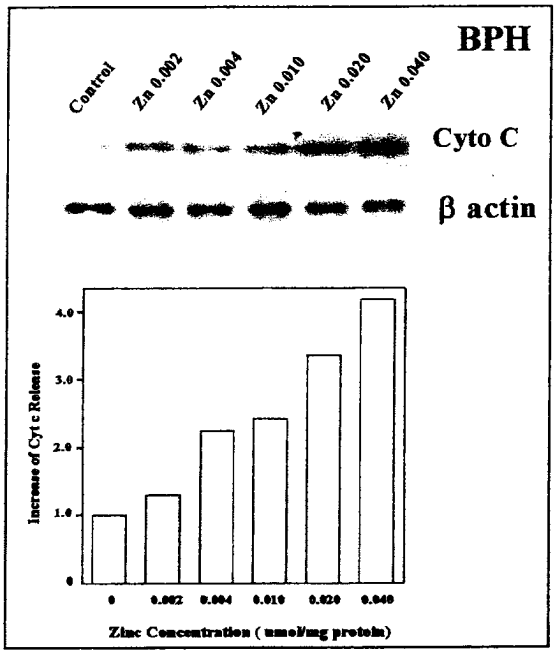


Fig. 5. Zinc induction of mitochondrial cytochrome c release in BPH cell-free system is concentration dependent. Mitochondria prepared from BPH cells were incubated with zinc at the concentrations indicated for 20 min at 30°C. The cytochrome c (Cyto c) release was determined as described in Figure 4.

ability of cells to accumulate high zinc levels is a requirement for the induction of apoptosis by exposure to extracellular zinc (i.e., plasma levels).

However, the present studies reveal another significant relationship. The issue raised is whether the lack of a pro-apoptotic effect of zinc on HPR-1 cells is due to an insufficient level of intracellular zinc and/or due to a lack of responsiveness of the mitochondria to zinc. Under the same experimental conditions that induced the release of cytochrome c from isolated PC-3 and BPH mitochondria, the isolated mitochondria from HPR-1 cells were not responsive to the direct exposure to zinc. It is now evident that the cell-specific effects of zinc induction of mitochondrial apoptogenesis as demonstrated in this and earlier reports [5,6] are dependent upon two factors: (1) the ability of cells to accumulate sufficiently high levels of mobile

Fig. 4. Zinc induction of mitochondrial cytochrome c release in prostatic cell-free system. Mitochondria were prepared from PC-3 cells (**A**), BPH cells (**B**), HPR-1 cells (**C**) as described in the Materials and Methods section and were incubated with or without zinc (0.01 – 0.02 μM/mg protein) for the time periods indicated. The levels of released cytochrome c determined by Western blot are presented by the relative O.D. from densitometry scan of the cytochrome c (Cyto c) bands corrected by the values of β actin as internal controls.

reactive zinc necessary to induce mitochondrial release of cytochrome c; and (2) the ability of mitochondria from different cells to respond to the direct effect of zinc. An important issue that has to be pursued is the reason for the different zinc-responsivenesses of mitochondria from different cells. In this regard, we have shown that zinc-altered mitochondrial aconitase equilibrium is unique to prostate mitochondria due to a specific protein not associated with other mitochondria [11].

A direct effect of zinc on the mitochondrial release of cytochrome c was evident at zinc concentrations as low as 7.6 μM (0.004 μmol zinc/mg mitochondrial protein). Although the free zinc concentrations of mammalian cells is unknown, the cytosolic pool of free zinc has been estimated to be 10^{-5}–10^{-12} M and even as low as 10^{-15} M [12,13]. The total cellular zinc has been calculated to be in the range of approximately 10^{-3} M. These values are likely higher for prostate cells, because prostate accumulates approximately three to five times more zinc than other tissues. Moreover, cellular reactions of zinc are dependent upon mobile reactive zinc, not solely free zinc, through intermolecular exchange of zinc. This pool of zinc, although its cytosolic concentration is unknown, is magnitudes greater than the free zinc pool. Consequently, the effectiveness of zinc levels in the range of 10^{-6} M zinc as used in this study likely is representative of in situ cytosolic reactive zinc levels and is, therefore, a physiological effect of zinc in the prostate cells.

The present studies combined with our previous reports demonstrate a consistent and persistent effect of zinc in the induction of mitochondrial apoptogenesis in specific prostate epithelial cells. This effect is observed when the cells are exposed to conditions that are representative of the plasma level of zinc (approximately 2–15 μM). In contrast, most reports with other mammalian cells describe a role of zinc in the inhibition of apoptosis [14,15]. In the overwhelming majority of those reports, the levels of zinc to which the cells were exposed were in the millimolar range, i.e., unphysiological concentrations to which cells in situ would never be exposed. This, in part, could account for the difference in the zinc effects on apoptosis. This concern is also applicable to the report that zinc induces necrosis in prostate cells. In that study, 200–500 μM zinc (conditions that would never exist in situ) was required to induce necrosis; and the necrotic effect was not evident at lower, but still unphysiologic, levels of zinc [16]. However, similar to our observations with prostate cells, a few reports exist that also demonstrate zinc induction of apoptosis in select mammalian cells [17–19]. In support of our observations, Untergasser et al. recently reported that zinc induces mitochondrial alterations in prostate cells that result in apoptosis [20]. The divergent reported effects of zinc as an inhibitor of apoptosis in some cells or conversely a stimulator of apoptosis in other cells might be due, in part, to the ability or lack thereof of the mitochondria in different cells to respond to the direct apoptogenic effect of zinc as demonstrated in this present study. Consequently, the generalization that zinc is a physiological inhibitor of apoptosis in mammalian cells is not tenable. Recognition of the cell-specific role of zinc in apoptosis and cell growth is essential. Moreover, our earlier report combined with this present report with prostate cells provides the first demonstration in any mammalian cells (to our knowledge) that the zinc-induced apoptotic effect is targeted directly at the induction of mitochondrial apoptogenesis.

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Human ZIP1 is a major zinc uptake transporter for the accumulation of zinc in prostate cells

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Abstract

The prostate gland of humans and other animals accumulates a level of zinc that is 3–10 times greater than that found in other tissues. Associated with this ability to accumulate zinc is a rapid zinc uptake process in human prostate cells, which we previously identified as the hZIP1 zinc transporter. We now provide additional evidence that hZIP1 is an important operational transporter that allows for the transport and accumulation of zinc. The studies reveal that *hZIP1* (SLC39A1) but not *hZIP2* (SLC39A2) is expressed in the zinc-accumulating human prostate cell lines, LNCaP and PC-3. Transfected PC-3 cells that overexpress *hZIP1* exhibit increased uptake and accumulation of zinc. The V_{max} for zinc uptake was increased with no change in K_m. Along with the increased intracellular accumulation of zinc, the overexpression of *hZIP1* also results in the inhibition of growth of PC-3 cells. Down-regulation of hZIP1 by treatment of PC-3 cells with hZIP1 antisense oligonucleotide resulted in a decreased zinc uptake. Uptake of zinc from zinc chelated with citrate was as rapid as from free zinc ions; however, the cells did not take up zinc chelated with EDTA. The cellular uptake of zinc is not dependent upon an available pool of free Zn^{2+} ions. Instead, the mechanism of transport appears to involve the transport of zinc from low molecular weight ligands that exist in circulation as relatively loosely bound complexes with zinc.

Keywords: Zinc; Zinc transporter; Prostate cells; ZIP transporter

1. Introduction

The prostate gland accumulates high levels of zinc. The tissue level of zinc in prostate is 3–10 times greater than that found in other tissues. It is this ability of the prostate epithelial cells to accumulate high zinc levels that results in the major prostate functions of citrate accumulation and secretion. Citrate accumulation results from inhibition of mitochondrial (m)-aconitase by zinc that prevents the oxidation of citrate via the Krebs cycle [1,2]. In addition, the accumulation of zinc also results in the inhibition of growth of prostate cells through its stimulation of mitochondrial apoptogenesis [3]. Consequently, it is evident that a significant component of the high intracellular level of zinc is in the form of reactive zinc.

While zinc is an important co-factor for many enzymes and cellular proteins and is essential for the normal function of all cells, only recently have we begun to understand the mechanisms of cellular zinc uptake by mammalian cells. The existence of a rapid zinc-uptake transport process has been reported for the human prostate cell lines, LNCaP and PC-3 [4]. We identified *hZIP1* (SLC39A1) as a possible zinc transporter responsible for zinc uptake and accumulation in these cells. Transporters that transport zinc across the plasma membrane from the extracellular fluid have been identified in *Saccharomyces cerevisiae* [5,6] and *Arabidopsis* [7]. These transporters are members of the ZIP (Zrt/Irt-like proteins) family. In *Arabidopsis* three genes have been identified, *Zip1*, *Zip2* and *Zip3*. Four members of the *Zip* family, *ZIP1*, *ZIP2*, *ZIP3* and *ZIP4* have also been identified in mammalian cells [7–9]. Gaither and Eide [10] indicated that hZIP1 is the likely endogenous uptake transporter in mammalian cells. In the present report we provide compelling evidence that hZIP1 is an important functional transporter that is responsible for the rapid uptake and accumulation of

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physiologically effective zinc in prostate cells and suggest a mechanism of cellular zinc transport.

2. Experimental

2.1. Cell culture methods and transfection

PC-3 cells (from ATCC) were grown in complete DMEM medium supplemented with 10% fetal bovine serum (Gibco). Cells were plated in T 75 flasks, incubated in a humidified atmosphere of 5% CO_2 and air and transfected with 10 μg of pRc-CMV or pCMV-hZIP1 vectors [10] using Effectene reagents (Qiagen). Stably transfected clones were selected by the dilution plating technique in 350 μg/ml of G418. The stable transfectant cell lines were maintained in 350 μg/ml G418.

2.2. ^{65}Zn uptake assay

Cells were grown to 70–75% confluence, harvested by trypsin digestion from the flasks, washed once in cold Hanks Balanced Salt Solution (HBSS) and counted by hemacytometer. The cells were resuspended in cold uptake-buffer (HBSS, 50 mM HEPES, pH 7.4) and maintained on ice. Uptake assays were initiated by addition of 100 μl of cell suspension to 150 μl of pre-warmed uptake-buffer containing the specified concentration of $ZnCl_2$ and a constant specific activity of ^{65}Zn (Amersham Pharmacia Biotech, Inc.). The cell suspension was incubated at 37 °C unless otherwise indicated. Assays were terminated by addition of four volumes (1 ml) of stop-buffer (50 mM HEPES, 250 mM sucrose, 1 mM EDTA, pH 7.2). Cells were collected by filtration through glass fiber filters (Whatman GF/C) on a Brandel cell harvester. The filters were washed six times with wash-buffer (1×PBS with 1 mM EDTA) and counted by liquid scintillation.

2.3. RT-PCR analysis

hZIP and *GAPDH* cDNA were synthesized from total mRNA isolated from human prostate cells using 1.0 μg of total RNA, reverse transcriptase and random primers (TaqMan7 reagents, Perkin Elmer). *hZIP1, hZIP2* and *GAPDH* fragments were amplified from the cDNA using 1.0 μM forward and reverse primers and 35 cycles. These conditions were shown to be in the quantitative detection range based on the concentration of template DNA. The cloned cDNA for *hZIP1* and *hZIP2* was used as the template DNA in control reactions to determine the specificity of the PCR reactions. The RT-PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining and photographed under UV light. No products were detected without reverse transcriptase. The primers for *hZIP1* were 5′-TCAGAGCCTCCAGTGCCTGT-3′ and 5′-GCAGCAGGTCCAGGAGACAA-3′; the primers for *hZIP2* were 5′-TGGTTCCAGATTGATGCAGC-3′ and 5′-CTGATCTGTTCTGCACCATG-3′; and the GAPDH primers were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′.

2.4. Anti-hZIP1 antibody production and immunoblotting

Chicken polyclonal antibodies were raised against a peptide corresponding to residues 133–146 and affinity purified using the same peptide. Antibody depleted and preimmune preparations were used to verify the specificity of the antibody. Total protein extracts were prepared from cells in lysis buffer (25 mM HEPES, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1.0% NP-40) and the protein concentration determined using the BioRad protein assay based on the Bradford procedure [11]. Equal amounts of protein were subjected to SDS–PAGE gel electrophoresis according to the method of Laemmli [12], transferred to nitrocellulose membranes and incubated overnight with anti-hZIP1 antibody. Protein-bound antibody was detected using enhanced chemiluminescence detection reagents from the Amersham Corporation. The membranes were then stripped of antibody and re-probed with anti-β-actin antibody.

2.5. Antisense oligonucleotide inhibition of hZIP1 expression

Sense and antisense oligodeoxyphosphorothionucleotides were designed to bind to regions of hZIP1 and hZIP2 mRNA that include the start codon. The sequences for *hZIP1* and *hZIP2* were 17 bases long and were from location −5 to 12 relative to the ORF. The sense sequences were 5′-GCATCATGGGGCCCTGG-3′ and 5′-CAGAGATGGAGCAACTA-3′ and the antisense sequences were 5′-CCAGGGCCCCATGATGC-3′ and 5′-TAGTTGCTCCATCTCTG-3′ for *hZIP1* and *hZIP2*, respectively.

2.6. Assessment of intracellular zinc

CMV and *hZIP1* transfected PC-3 cells were cultured under normal culture conditions in medium containing 350 μg/ml of G418. After the cells reached 75% confluence, the medium was removed, the cells washed with 1×PBS (pH 7.4) and incubated in 20 μM Zinquin Ester for 30 min. The dye solution was removed and the cultures washed three times with 1×PBS. The cultures were viewed and digital images captured using an inverted fluorescence microscope and UV filters.

3. Results

3.1. hZIP1 expression in prostate cells

We previously reported that the rapid uptake of zinc by LNCaP cells was greater than that of PC-3 cells and that both cell lines expressed hZIP1 [4]. Therefore, we wanted to determine if differences in the expression of a zinc transporter could account for this difference in zinc uptake. Fig. 1 shows the results of RT-PCR reactions for *hZIP1* and *hZIP2* using total RNA isolated from LNCaP and PC-3 cells. The results show a higher level of *hZIP1* expression in LNCaP compared with PC-3 cells. In addition, these results also show that expression of *hZIP2* was undetectable under these conditions. To assess the specificity of the PCR primers we used the cloned cDNAs for *hZIP1* and *hZIP2* as templates in PCR reactions carried out in the same microwell plates as the RNA samples. Results of these reactions showed that the primers amplified fragments that corresponded to the appropriate *ZIP* transporter. More importantly, the results showed that the lack of amplification of *hZIP2* from mRNA was not due to the primers or PCR conditions.

3.2. Overexpression of hZIP1 in pc-3 cells

PC-3 cells were transfected with pCMV-hZIP1 or pRc-CMV (control) as described in the Experimental section. *hZIP1* expression was assayed by RT-PCR and by Western blots and the accumulation of ^{65}Zn in hZIP1 transfected cells and CMV vector-only control cells measured. Fig. 2a shows the expected 212-bp fragments from mRNA samples and the overexpression of *hZIP1*. Fig. 2b shows hZIP1 protein levels in cells overexpressing hZIP1 compared with CMV controls.

Fig. 3 shows that overexpression of hZIP1 resulted in an increase in the uptake of ^{65}Zn. The kinetics of zinc transport showed an increase in V_{max} for cells overexpressing hZIP1 compared with the CMV control (105±7.8 and 46±1.9 pmol/min per 10^6 cells, respectively) with no significant change in the K_m (7.6±2.3 and 6.6±2.3 μM).

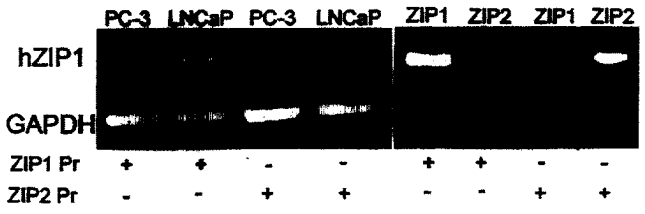

Fig. 1. Expression of endogenous *hZIP* transporter in LNCaP and PC-3 cells. ZIP1 and ZIP2 lanes show PCR products where cloned ZIP1 or ZIP2 cDNA was the template with each primer pair. PC-3 and LNCaP lanes show RT-PCR products using total RNA from cell extracts. ZIP1 Pr and ZIP2 Pr indicate the primers used in each PCR reaction. GAPDH was assayed to ensure approximately equal template concentrations. No PCR products were obtained without reverse transcriptase.

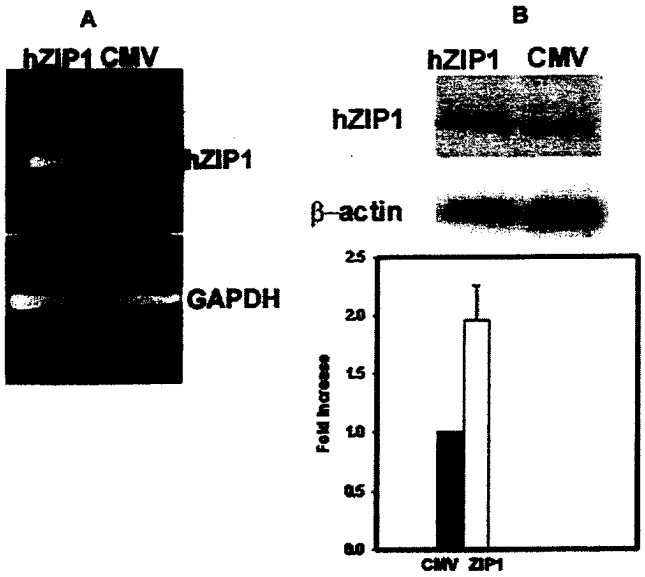

Fig. 2. Overexpression of hZIP1 mRNA and protein. (A) RT-PCR analysis of hZIP1 and GAPDH mRNA extracted from PC-3 cells stably transfected with hZIP1 or the CMV control vectors. (B) Upper panel shows Western blot analysis of hZIP1 and β-actin from cell extracts of hZIP1 transfected and CMV control PC-3 cells. Bottom panel shows quantification of Western blot. hZIP1 transfected cells had approximately two fold more hZIP1 than the CMV control.

These kinetics are consistent with an increase in the abundance of hZIP1.

To determine if the overexpression of *hZIP1* resulted in the increased accumulation of cellular zinc, we incubated over-expressing PC-3 cells and CMV control cells with ^{65}Zn for various periods. Cells transfected with *hZIP1* accumulated more zinc than the CMV transfected controls (Fig. 4). In the same experiments we also measured ^{65}Zn accumulation at 4 °C. The accumulation of ^{65}Zn at the lower temperature was greatly decreased in both the CMV controls and the hZIP1 over-expressing cells indicating that the uptake process was temperature-dependent and

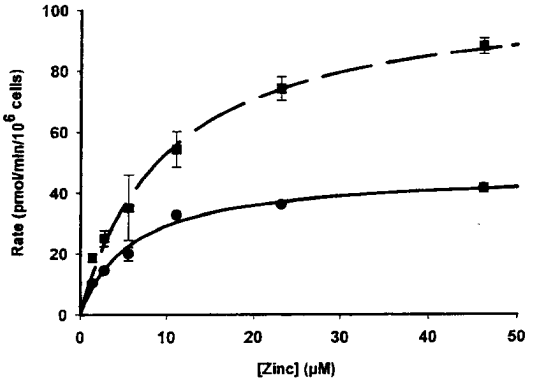

Fig. 3. Concentration dependent zinc uptake. Zinc uptake kinetics of CMV control (circles) and hZIP1 transfected (squares) cells. Control and hZIP1 cells were cultured under identical conditions, collected and uptake of ^{65}Zn assayed as described in the Experimental section.

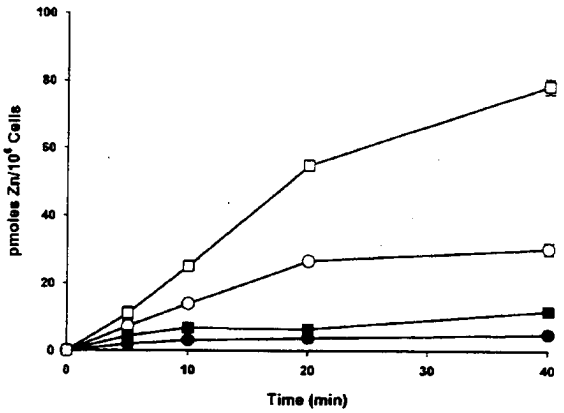

Fig. 4. Time dependent zinc uptake. Zinc accumulation by hZIP1 (squares) and CMV control transfectants (circles) measured at 37 °C (open symbols) and at 4 °C (closed symbols). Cells were cultured under identical conditions, collected and zinc accumulation measured from 0 to 40 min at a medium zinc concentration of 15 μM $ZnCl_2$. Values are means±S.E.M. of a representative experiment ($n=3$).

likely a transport process. To ensure that the accumulated zinc was intracellular and not zinc that was bound to the cell membrane we used Zinquin to visualize intracellular zinc. Cells transfected with *hZIP1* showed an intracellular increase in Zinquin fluorescence that indicated an increase in cellular zinc (Fig. 5).

3.3. Antisense inhibition of hZIP1 expression

Since RT-PCR assays showed that *hZIP1* was expressed in LNCaP and PC-3 cells and that *hZIP2* was undetectable, we determined if inhibition of *hZIP1* expression would decrease the accumulation of zinc by wt PC-3 cells. Sense and antisense oligonucleotides were used to inhibit *hZIP1* expression and ^{65}Zn uptake determined. Fig. 6A shows that cells incubated for 3 days in the presence of antisense *hZIP1* oligonucleotide exhibited a decrease in ^{65}Zn uptake compared with cells incubated in the presence of the sense oligonucleotide. To ensure that the treatment with the

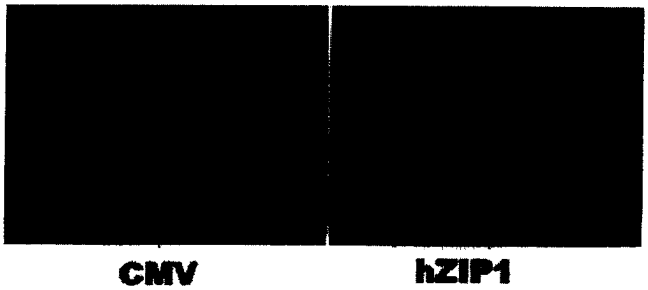

Fig. 5. Intracellular zinc in CMV (control) and hZIP1 PC-3 cells. Cells were cultured in complete medium, washed with PBS, incubated for 30 min with 20 μM Zinquin, washed again with PBS and viewed on an inverted fluorescence microscopy. Zinquin detectable zinc is localized in the cytoplasm around the nuclei. Arrowheads show the difference in cytosol florescence intensity in CMV compared to hZIP1 overexpressing cells.

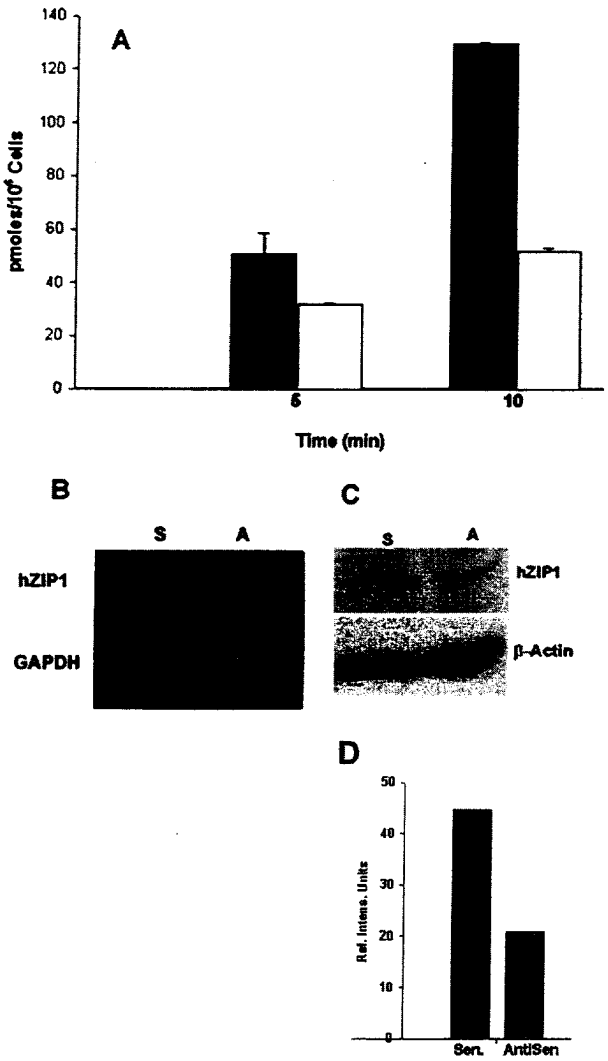

Fig. 6. Antisense oligonucleotide inhibition of hZIP1 expression and zinc accumulation. A shows zinc accumulation at 5 and 10 min by non-transfected PC-3 cells treated for 3 days with *hZIP1* sense (closed bar) and antisense (open bar) oligonucleotides. Each bar is the mean and S.E.M. for combined data from two experiments ($n=6$). B shows RT-PCR analysis of hZIP1 mRNA from cells cultured and treated under identical conditions as in A. C shows the Western blot analysis of hZIP1 protein levels of cells cultured and treated under identical conditions as A. D shows the quantification of the Western blot.

oligonucleotide decreased *hZIP1* expression, hZIP1 mRNA was measured by RT-PCR and hZIP1 protein was measured by Western blot after incubation with the sense and antisense oligonucleotides. Results showed that the antisense oligonucleotide was effective in decreasing the expression of *hZIP1* (Fig. 6b–d).

3.4. ^{65}Zn uptake in the presence of citrate and EDTA

The K_m value for the hZIP1 transporter is approximately 7 μM. The concentration of total zinc in blood plasma is 15–20 μM; but the concentration of free Zn^{2+} ion is estimated to be in the pM to nM range. Therefore, virtually

all of the zinc in circulation is bound to various ligands (i.e. Zn ligands) [13]. We wanted to determine if the PC-3 cells would take up zinc from Zn ligands as effectively as free Zn^{2+} ions. To achieve this, ^{65}Zn uptake was determined when zinc was chelated with citrate (ZnCit) and with EDTA (ZnEDTA) as compared to the uptake in the presence of $ZnCl_2$. Citrate or EDTA was added to 20 μM $ZnCl_2$ at a ligand/Zn ratio of 3:1, which provides an excess of ligand that ensures the virtual absence of any free Zn^{2+} ions with EDTA and greatly reduced Zn^{2+} ions with citrate. The results (Fig. 7a) reveal that the transport of zinc in the presence of ZnCit was as effective as the transport from $ZnCl_2$. In contrast, no zinc transport occurred from ZnEDTA. The 3:1 citrate/Zn ratio significantly decreased the concentration of Zn^{2+} ion; however, based on the formation constant (log K_f~5) for Zn citrate the concentration of free Zn^{2+} ion would still be approximately 4.0 μM under these conditions. To determine zinc transport at lower concentrations of free Zn^{2+} ion in the presence of ligand, we determined ^{65}Zn accumulation with increasing ratios of citrate/Zn. Under these conditions the total zinc concentration remained constant at 20 μM while the free Zn^{2+} ion concentration decreased with increasing citrate/Zn ratios. The results (Fig. 7b) show that the rate of zinc accumulation was not strongly dependent on the free Zn^{2+} ion concentration. Fig. 7c shows the uptake of zinc expressed as a percent of the maximum uptake rate for $ZnCl_2$ and $ZnCl_2$ in the presence of citrate. In the presence of citrate the concentration of total zinc remains constant as the concentration of free

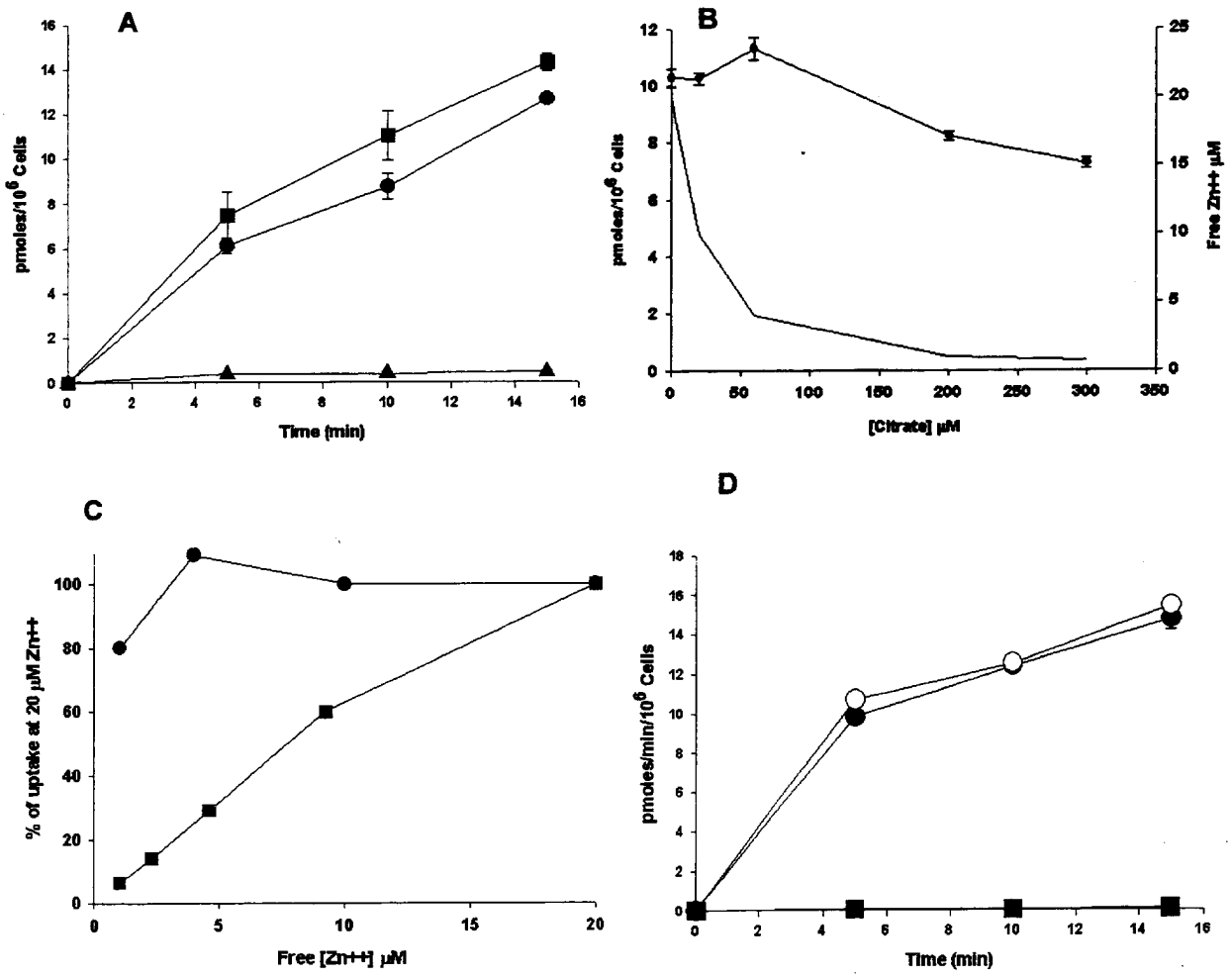

Fig. 7. Zinc accumulation in the presence of citrate and EDTA. A shows ^{65}Zn accumulation by PC-3 cells measured in the presence of $ZnCl_2$ (circles), ZnCit, (squares) and ZnEDTA (triangles). The concentration of zinc was 20 μM in the presence of 60 μM citrate or EDTA. Each point is the mean±S.E.M. for a representative experiment (n=3). B shows ^{65}Zn accumulation (circles) by PC-3 cells measured in the presence of increasing ratios of citrate/Zn. The concentration of Zn is constant at 20 μM while the citrate concentration increases from 0 to 300 μM. Each point is the mean±S.E.M. for a representative experiment (n=3). The solid line is the calculated concentration of free Zn^{2+} ion at each citrate to zinc ratio. C shows zinc uptake as a percent of the maximum uptake in the presence of $ZnCl_2$ alone (squares) and $ZnCl_2$ (20 μM) with increasing concentrations of citrate added (circles). Free [Zn^{2+}] on the x-axis presents the total available zinc with $ZnCl_2$ alone and the calculated free [Zn^{2+}] with citrate added. D shows the accumulation of ^{65}Zn (circles) and ^{14}C-citrate (squares) in the presence of $^{65}ZnCl_2$ (closed circles) and $^{65}Zn^{14}C$-citrate (open circles). Each point is the mean±S.E.M. for a representative experiment (n=3).

Zn^{2+} ion decreases with increasing citrate. The figure shows that the rate of zinc uptake at low concentrations of free Zn^{2+} ion, when free Zn^{2+} ions represent the total pool of zinc, is much lower than that at a comparable concentration of free Zn^{2+} ion in the presence of ZnCit complex. To determine if the uptake of zinc from ZnCit was due to transport of citrate rather than zinc transport, we determined the uptake of citrate when zinc was chelated with ^{14}C-citrate (Zn^{14}C-citrate). The results (Fig. 7d) showed no uptake of ^{14}C-citrate while the transport of zinc was again as effective as the transport from $ZnCl_2$. Therefore, it is evident that the transport of zinc is not dependent upon the availability of a free Zn^{2+} ion pool, and that the transporter can effectively transport zinc from a Zn ligand pool. However, since the formation constant is much higher for ZnEDTA (log K_f~16) than for ZnCit, the availability of transportable zinc appears to be dependent upon the relative binding affinities of the Zn ligand and the hZIP1 transporter.

3.5. Effect of hZIP1 overexpression on pc-3 cell growth

We reported that zinc inhibited the growth of PC-3 cells [14,15]. Therefore, we wanted to determine if overexpression of *hZIP1* and the resultant higher rate of zinc uptake would affect the growth of PC-3 cells. Fig. 8 shows the cell number with time of culture for the CMV controls and the *hZIP1* overexpressing cells. Cells that overexpress hZIP1 had slower growth rates than the CMV control. We also determined the effect of zinc added to the culture medium on cell number for CMV controls and cells overexpressing *hZIP1*. Fig. 9 shows that treatment with 15 μM $ZnCl_2$ resulted in a decrease in cell number of approximately 50% for the CMV control in 24 h and the loss of cells increased to approximately 70% by 72 h. The effect of zinc on cell number in cells overexpressing hZIP1

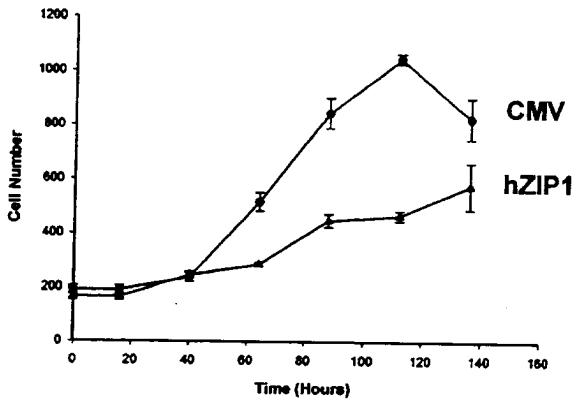

Fig. 8. hZIP1 over expression inhibits growth of PC-3 cells. CMV control and CMV-hZIP1 transfected cells were plated in multi well plates. The cells were cultured under standard conditions, collected at various times over a 136-h interval and the cell number determined. Each point is the mean±S.E.M. for combined results from two experiments (n=6).

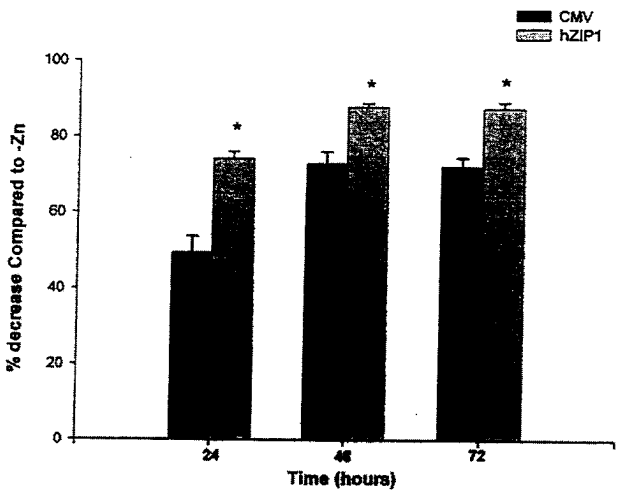

Fig. 9. hZIP1 overexpression increases the growth inhibitory effect of zinc. CMV and CMV-hZIP1 transfected cells were plated into multi well plates and cultured overnight under standard conditions. The medium was changed to serum free medium containing 15 μM zinc. The cells were incubated for 24, 48 and 72 h, collected and the cell number determined. Bars show the % decrease in cell number of zinc treated cultures compared with no zinc treatment controls. *Statistically different; CMV vs. hZIP1, P<0.05.

was significantly greater at each time point compared with the CMV control with a maximum decrease of approximately 90% by 72 h.

4. Discussion

We previously reported that the PC-3 and LNCaP prostate cell lines, which are zinc-accumulating cells, take up zinc with kinetics consistent with the presence of a plasma membrane rapid zinc uptake transporter [4]. Here we provide additional evidence that the transporter is the hZIP1 human zinc transporter. *ZIP1* and *ZIP2* are two members of the ZIP family identified in mammalian cells. In our earlier report we showed *hZIP1* expression in prostate cell lines by Northern blot. However, in that report we did not measure *hZIP2*. Here we measured the expression of hZIP1 and hZIP2 mRNA in LNCaP and PC-3 cell lines. The results showed that hZIP2 mRNA was not detectable in either LNCaP or PC-3 cells. Moreover, the expression of *hZIP1* in LNCaP cells was greater than that in PC-3 cells. These results are consistent with kinetic data that show a greater V_{max} for zinc uptake in LNCaP cells than PC-3 cells [4].

Additional evidence that hZIP1 is the endogenous zinc uptake transporter in prostate cells is obtained by the overexpression studies and the inhibited expression studies. Overexpression of *hZIP1* results in an increase in the zinc uptake rate and zinc accumulation by the prostate cells. This is accompanied by a corresponding increase in V_{max} but no change in the K_m value from that of the CMV control cells. Moreover, the increase in the level of hZIP1

expression in overexpressing cells was twofold, which corresponded to the increase in V_{max} of overexpressing cells compared with the CMV controls. Little zinc accumulation was observed at 4 °C, which is consistent with it being a transport effect. Exposure of wild type PC-3 cells to *hZIP1* antisense results in down-regulation of expression of *hZIP1* and a corresponding decrease in the transport of zinc.

There also exists a correlation between the increased expression of *hZIP1* and the cellular effects of the uptake and accumulation of zinc. The accumulation of zinc in prostate cells results in mitochondrial apoptogenesis due to zinc-induced release of cytochrome *c* [3]. Therefore, the negative relationship between the overexpression of zinc transport protein and the growth of the cells is consistent with an increase in the hZIP1-mediated zinc accumulation. We have shown that exposure of prostate cells to zinc-supplemented medium results in inhibition of growth due, in part, to increased mitochondrial apoptogenesis [3,15]. In accordance with this effect, the cells that overexpress *hZIP1* and transport more zinc also exhibit a greater zinc inhibition of growth.

Gaither and Eide [10] transfected K562 human erythroleukemia cells with *hZIP1* and showed that the characteristics of zinc transport in the transfected cells were identical to those in wild type cells. They concluded that hZIP1 is the endogenous plasma membrane transporter in K562 cells. Milon et al. [16] using immunofluorescence reported that hZIP1 was localized to the plasma membrane in K562 cells, in contrast the transporter was localized to intracellular vesicles in epithelial cells, including PC-3. They concluded that there is differential subcellular localization of hZIP1 in adherent and non-adherent cells. They proposed that hZIP1 is not likely to be involved with the uptake of zinc although they conducted no transport studies. Based on our previous report and the studies presented in this report, it is evident that hZIP1 is associated with the rapid net uptake of zinc from the medium by prostate cells, resulting in a net increase in the intracellular level of zinc. Therefore, hZIP1 operates as a rapid zinc-uptake transporter and the increase in cellular zinc could not be the result of intracellular zinc translocation and sequestration.

The issue of the physiological importance of the hZIP1 transporter for zinc accumulation in prostate cells needs to be addressed. It must be recognized that the plasma membrane of the cell is subjected to the conditions of the interstitial fluid (ISF), which is an ultrafiltrate of the blood plasma. Therefore, zinc is present in ISF bound to low molecular weight (LMW) zinc ligands and as free Zn^{2+} ions with very little protein bound zinc. Since the free Zn^{2+} ion concentration is 1000-fold lower than the hZIP1 K_m (approx. 7 μM), it is most unlikely that free Zn^{2+} is the source of zinc for rapid transport and cellular accumulation. Nevertheless, Gaither and Eide [10] proposed that hZIP1 transport involved the transport of zinc from a free Zn^{2+} ion pool as a result of the high capacity of the transporter. The current results (Fig. 7a) demonstrate that the transport of zinc from ZnCit is as effective as the transport from free zinc ions. The fact that zinc was not transported from ZnEDTA (Fig. 7a) under the same conditions as with ZnCit indicates that the binding affinity of the Zn ligand is an important determinant in the transportability of zinc by hZIP1. This suggests that, functionally, hZIP1 transports zinc from the LMW Zn ligand pool (e.g. amino acids, citrate, others) that exists in plasma and ISF. The concentration of this zinc pool would approximate the K_m value of the hZIP1 transporter.

Since free Zn^{2+} ion is such a small fraction of the total zinc present in plasma, others have suggested that LMW Zn ligands are the transported forms of the metal [13]. However, the results reported here demonstrate that the citrate component of the ZnCit complex was not transported into the cells. Thus, the current results indicate that zinc was not transported as a LMW complex. These observations indicate that the mechanism of zinc transport involves an intermolecular exchange of zinc between Zn ligand and the hZIP1 transporter. We are now extending these studies to establish the validity of this proposed mechanism and to define the structural–functional relationship of hZIP1.

In summary this report, coupled with our previous studies, demonstrates three important features of zinc uptake and accumulation in prostate cells. First, hZIP1 is an important functional zinc uptake transporter in prostate cells. Second, the hZIP1-mediated uptake results in the cellular accumulation of zinc that alters the growth and metabolic activities of the cell. Third, the transporter is not limited for transport to a pool of free Zn^{2+} ions; but, more likely, transports zinc from the available low molecular weight zinc-ligand pool that exists in plasma and ISF through an hZIP1–Zn ligand intermolecular exchange of zinc.

5. Abbreviations

hZIP	human ZIP
RT-PCR	reverse transcription-polymerase chain reaction
PBS	phosphate-buffered saline
CMV	cytomegalovirus
ORF	open reading frame
DMEM	Dulbecco's Modified Eagle medium
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldeyde-3-phosphate dehydrogenase
G418	geneticin selective antibiotic
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
PMSF	phenylmethylsulfonyl fluoride
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

Acknowledgements

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Short Communication

Metallothionein can function as a chaperone for zinc uptake transport into prostate and liver mitochondria

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Abstract

In mammalian cells the cytosolic concentration of free Zn^{2+} ions is extremely low (nM–fM range) and unlikely to provide an adequate pool for the uptake and accumulation of zinc in mitochondria. We previously identified a mitochondrial uptake transport process that effectively transports zinc directly from low molecular weight zinc ligands independent of and in the absence of available free Zn^{2+} ions. Since metallothionein (MT) is an important ligand form of cellular zinc, we determined if Zn_7-MT was an effective chaperone and donor for delivery and uptake of zinc by prostate and liver mitochondria. The results reveal that both intact mitochondria and mitoplasts effectively accumulated zinc from Zn_7-MT. The study confirms and extends our previous report that the putative zinc transporter is associated with the inner mitochondrial membrane and involves a direct exchange of zinc from the ligand to the transporter. The ventral prostate cells contain no detectable MT; so that ligands (such as citrate, aspartate) other than MT are zinc donors for mitochondrial zinc accumulation. However, in liver and perhaps other cells, Zn_7-MT is probably important in the cytosolic trafficking of zinc to the mitochondria for the uptake of zinc into the mitochondrial matrix by the putative zinc uptake transporter.

Keywords: Zinc uptake transporter; Mitochondria; Zinc metallothionein; Prostate; Liver

1. Introduction

We previously reported the kinetic identification of a putative zinc uptake transporter in prostate and liver mitochondria [1]. The transporter is associated with the inner mitochondrial membrane, exhibits the characteristics of a facilitative transport process, does not require a free Zn^{2+} ion pool for transport; and effectively transports zinc from zinc ligands with $\log K_f$ values up to ~11, but not from ligands with $\log K_f$ values equal to or greater than 13. The transporter exhibits an apparent $\log K_f \sim 12$. The Km ranges from about 30–80 μM zinc, depending upon the ligand and the source of mitochondria. Because the free Zn^{2+} ion concentration of cytosol is negligible (nanomolar to femtomolar range) [2], we postulated that low molecular weight zinc ligands (ZnLigands) comprise the major cytosolic pool of zinc for delivery to and transport into the mitochondria by this putative zinc uptake transporter.

It was essential to determine if zinc metallothionein might also be an important donor form for mitochondrial zinc uptake. Recently we obtained two different Zn_7-MT sources with which we determined zinc transport into mitochondria. Rabbit liver Zn_7-MT-2, prepared as described in Halthout et al. [3], was kindly provided by Dr. C. Fenselau at the University of Maryland, College Park, MD. Recombinant human Zn_7-MT, described in Hong and Maret [4], was generously provided by Dr. Maret at the University of Texas Medical Branch, Galveston, Texas. Both preparations were devoid of free Zn^{2+} ions. A poly-clonal antibody against human and rat MT-1 and -2 proteins was generously provided by Dr. P.C. Huang at Johns Hopkins University, Baltimore, MD [5]. The methods for prostate and liver mitochondria preparation, the conditions

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for zinc accumulation, and all other assays were the same as previously described [1]. Although the experimental protocol was limited by the availability Zn_7-MT, the results obtained were apparent, conclusive and consistent with our earlier report [1].

Zinc uptake from 30 μM purified rabbit liver Zn_7-MT-2 (MT-2: 30 μM; total bound zinc: 210 μM) by intact mitochondria was compared to that from 30 μM ZnCysteine (zinc: 30 μM; Cysteine: 90 μM). The latter is included because we previously showed that it is as effective as free Zn^{2+} ions as a donor form for mitochondrial uptake and because its $\log K_f \sim 10$ is close to Zn_7-MT-2 (~11–12). Fig. 1(a) shows that mitochondrial zinc accumulation from rabbit liver Zn_7-MT-2 was as effective as from ZnCysteine in both liver and ventral prostate mitochondrial preparations. The results with ZnCysteine also confirms our previous observation that the liver and prostate mitochondria have the capacity to accumulate similar levels of zinc. In order to determine if zinc accumulation was due to the mitochondrial accumulation of Zn_7-MT-2, the levels of the mitochondrial MT were determined by Western blot. Fig. 1(b) shows that the presence of endogenous ~12 kDa MT in liver mitochondria, but no significant MT change was observed during incubation with rabbit liver Zn_7-MT-2. In the ventral prostate mitochondria, no detectable MT was found in the presence or absence of 30 μM rabbit liver Zn_7-MT-2. Therefore, zinc accumulation involves the transfer of zinc from the ligand, which confirms our previous observation [1] with other ZnLigands.

Zinc accumulation was also determined from recombinant Zn_7-MT (Maret preparation) in liver mitoplast preparation. Fig. 2(a) shows that significant zinc accumulation resulted from Zn_7-MT; but, unlike in Fig. 1(a), somewhat less than the accumulation from ZnCysteine. The absence of zinc accumulation from ZnEDTA shows that the inner membrane is in tact, and that ZnEDTA is not a zinc donor form due to its high binding affinity ($\log K_f \sim 16$) as we previously reported [1]. The absence of MT accumulation in the mitoplasts (Fig. 2(b)) confirms that zinc is transferred from the ligand in the transport process, and confirms that the putative transporter is associated with the inner membrane. An apparent difference in the recombinant preparation is its detection as a ~40 kDa doublet for which no explanation can be provided at this time (personal communication with Dr. Maret). This might also account for the lower zinc accumulation with this preparation. Nevertheless, when combined with the results in Fig. 1, it is apparent that Zn_7-MT-2 is an effective zinc donor for the putative zinc uptake

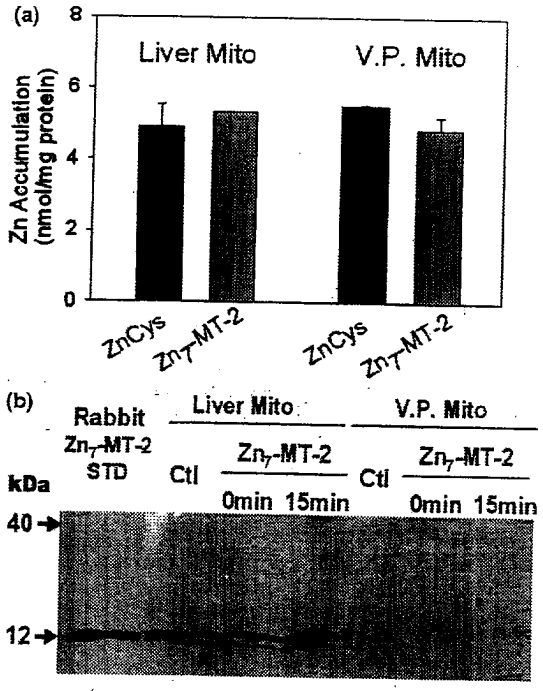

Fig. 1. Liver and VP mitochondrial zinc accumulation from rabbit liver Zn_7-MT-2: (a) comparison of zinc accumulation from ZnCys and Zn_7-MT-2 in liver and ventral prostate mitochondria by atomic absorption assay. ZnCys contains 30 μM zinc and 90 μM ligand. Zn-MT contains 30 μM MT and 210 μM zinc. Zinc uptake was performed for 10 min incubation at room temperature. (VP Zn_7-MT: $n = 2$; liver Zn_7-MT: $n = 1$; ZnCys: $n = 3$); (b) western blot detection of MT-2 imported into isolated liver and ventral prostate mitochondria. Aliquots of 50 μg mitochondrial protein were used for western blot. Purified rabbit liver MT-2 (0.1 μg) served as a MT standard.

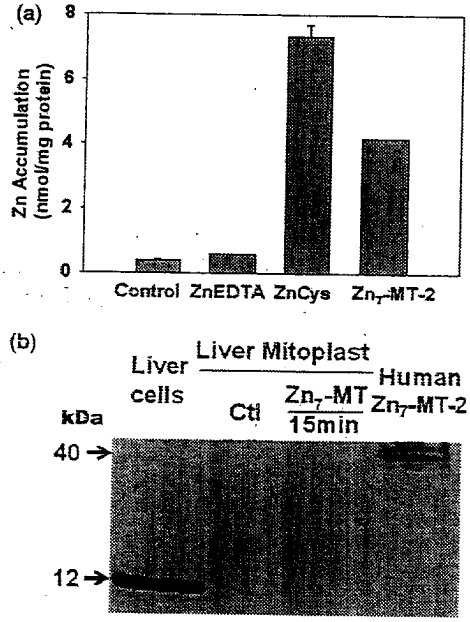

Fig. 2. Zinc accumulation from recombinant human Zn_7-MT-2 by liver mitoplast: (a) zinc accumulation from 30 μM ZnCys, Zn_7-MT-2 and ZnEDTA by liver mitoplast by atomic absorption assay. Conditions were the same as Fig. 1(a). (Zn_7-MT: $n = 2$; others: $n = 3$); (b) western blot detection of Zn_7-MT-2 imported into liver mitoplast. Aliquots of 50 μg mitoplast protein were used for western blot. Fifty microgram liver cell protein extract and 0.1 μg recombinant human MT-2 served as MT standards.

transporter involved in the transport of zinc across the inner membrane and into the mitochondrial matrix. The results are also consistent with our earlier conclusion that the putative zinc uptake transporter has an apparent $\log K_f \sim 12$.

Previous studies indicated that oxidants can induce zinc release from MT by oxidizing the sulfur ligands in zinc/thiolate cluster [6–9]. Ye et al. [10] reported that cytosolic Zn_7-MT-2 could enter the intermembrane space where oxidative conditions and/or lower pH cause the release of zinc that inhibits terminal oxidation. This raises the possibility that released free Zn^{2+} ions from Zn_7-MT-2 traversed the inner membrane into the mitochondrial matrix. However, our zinc uptake studies employed non-respiring oxidatively inactive in tact mitochondria and mitoplasts. Therefore the uptake of zinc from Zn_7-MT-2 did not require a release of free Zn^{2+} ions for transport into the mitochondria, as we previously reported [1] for other ligands. This is consistent with the observation that zinc is directly transferred from Zn_7-MT-2 to acceptor protein without the release of free Zn^{2+} ion [3]. Although a total of seven zinc binds to one MT molecule, previous studies demonstrated that Zn_7-MT does not transfer all of its zinc to other proteins, but one zinc in the Zn_3-β cluster is more prone to transfer than the others[3,8,11]. This might account for the observation (Fig. 1) that the accumulation of zinc from Zn_7-MT-2 that contains 210 μM zinc/30 μM MT-2 was similar to zinc accumulation from 30 μM ZnCysteine.

In the rat ventral prostate cells, the high levels of citrate and aspartate are the apparent important ZnLigands that serve as cytosolic zinc-donors for the characteristic high mitochondrial uptake and accumulation of zinc [1] since metallothioneins are not detectable in these cells [unpublished information; 11–14]. In liver, and likely other mammalian cells, Zn_7-MT-2 could be an important chaperone for delivery to and transport of zinc by the mitochondria. Most importantly, the mounting evidence supports the concept that, in the absence of a sufficient cytosolic free Zn^{2+} pool, loosely bound ZnLigands (including Zn_7-MT-2) are effective donors of zinc for the mitochondrial transport of zinc.

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Kinetic identification of a mitochondrial zinc uptake transport process in prostate cells

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Abstract

Prostate cells accumulate high cellular and mitochondrial concentrations of zinc, generally 3–10-fold higher than other mammalian cells. However, the mechanism of mitochondrial import and accumulation of zinc from cytosolic sources of zinc has not been established for these cells or for any mammalian cells. Since the cytosolic concentration of free Zn^{2+} ions is negligible (estimates vary from 10^{-9} to 10^{-15} M), we postulated that loosely bound zinc–ligand complexes (Zn-Ligands) serve as zinc donor sources for mitochondrial import. Zinc chelated with citrate (Zn-Cit) is a major form of zinc in prostate and represents an important potential cytosolic source of transportable zinc into mitochondria. The mitochondrial uptake transport of zinc was studied with isolated mitochondrial preparations obtained from rat ventral prostate. The uptake rates of zinc from Zn-Ligands (citrate, aspartate, histidine, cysteine) and from $ZnCl_2$ (free Zn^{2+}) were essentially the same. No zinc uptake occurred from either Zn-EDTA, or Zn-EGTA. Zinc uptake exhibited Michaelis–Menten kinetics and characteristics of a functional energy-independent facilitative transporter associated with the mitochondrial inner membrane. The uptake and accumulation of zinc from various Zn-Ligand preparations with log K_f (formation constant) values less than 11 was the same as for $ZnCl_2$, and was dependent upon the total zinc concentration independent of the free Zn^{2+} ion concentration. Zn-Ligands with log K_f values grater than 11 were not zinc donors. Therefore the putative zinc transporter exhibits an effective log K_f~11 and involves a direct exchange of zinc from Zn-Ligand to transporter. The uptake of zinc by liver mitochondria exhibited transport kinetics similar to prostate mitochondria. The results demonstrate the existence of a mitochondrial zinc uptake transporter that exists for the import of zinc from cytosolic Zn-Ligands. This provides the mechanism for mitochondrial zinc accumulation from the cytosol which contains a negligible concentration of free Zn^{2+}. The uniquely high accumulation of mitochondrial zinc in prostate cells appears to be due to their high cytosolic level of zinc-transportable ligands, particularly Zn-Cit.

Keywords: Zinc transport; Mitochondrial transport; Citrate; Prostate cells; Liver cells

1. Introduction

Prostate secretory epithelial cells have the function and capability of accumulating extremely high zinc levels, generally 3–10-fold higher than other mammalian cells; see [1–3] for recent reviews of zinc relationships in prostate. In addition to the high total cellular zinc level, the mitochondria of the prostate cells also accumulate high zinc levels. The accumulation of high zinc levels results in altered mitochondrial function such as inhibition of *m*-aconitase and citrate oxidation and induction of mitochondrial apoptogenesis [4,5]. Therefore, a significant component of the total cellular zinc must exist in a mobile reactive form.

Estimates place the total cellular zinc content of mammalian cells at approximately 0.2 mM. In prostate this value approximates 1–5 mM depending upon the source of prostate tissue (e.g. rat or human prostate). For this discussion we would define three pools of zinc that comprise the total cellular zinc: (a) tightly bound zinc (mainly metalloenzymes, metalloproteins, nucleoproteins) that is an immobile unreactive pool; (b) loosely bound zinc (such as amino acid and citrate bound) which constitute a mobile reactive pool; (c) free Zn^{2+} ion which would be a reactive pool. The immobile unreactive pool comprises

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>95% of the total cellular zinc. The free Zn^{2+} ion concentration is negligible; estimated to be in the nanomolar to picomolar range, and even as low of femtomolar concentration [6,7]. Therefore, zinc bound to mainly low molecular ligands (Zn-Ligands) comprises the major mobile reactive pool of zinc. Even if this pool represents as little as 0.1–1% of the total cellular zinc, its concentration would approximate 0.2–2 μM in mammalian cells; and about 1–10 μM in prostate cells.

These relationships raise the important question: 'How is zinc trafficked through the cytosol for uptake and accumulation in mitochondria of prostate cells?' Prostate cells, unlike other mammalian cells, contain a high cellular concentration of citrate which is a major ligand for zinc that represents as much as 30% of the total cellular zinc [8–10]. Since cytosolic free Zn^{2+} ion concentration is negligible (10^{-9}–10^{-15} M), we postulated that the mitochondrial accumulation of zinc must involve a mitochondrial zinc uptake process that is not dependent upon the transport of zinc from a cytosolic free Zn^{2+} ion pool. However, as of this time, the existence of a specific mitochondrial zinc uptake transporter or transport process has not been reported in any mammalian cells. We now present evidence that prostate mitochondria contain a specific zinc uptake transport process that involves a zinc exchange from donor Zn-Ligands to a putative zinc transporter located on the inner mitochondrial membrane; and that the transport does not require free Zn^{2+} ion transport. Preliminary evidence is presented that this zinc transport process also exists in liver mitochondria.

2. Experimental procedures

2.1. Isolation of mitochondria

Young adult male Wistar rats weighing between 300 and 350 g were employed as the source of tissues for these studies. The handling and treatment of animals were in accordance with the regulations and guidelines of the National Institutes of Health and the University of Maryland. The preparation of prostate and liver mitochondria has been described previously [4,5]. All procedures were carried out at 2–4 °C on ice. Generally, rat ventral prostate (VP) and liver tissue were chopped into 1 mm pieces in isolation buffer (250 mM sucrose, 10 mM HEPES and 1 mM EDTA, pH 7.35), homogenized in a motor-driven glass homogenizer, and centrifuged at 500×*g* for 5 min. The supernatant fluid was centrifuged for 7 min at 12 000×*g* and the resulting pellet was washed twice in isolation buffer containing 0.25% fatty acid-free BSA, and washed once in reaction buffer (250 mM sucrose, 10 mM HEPES and 5 mM KH_2PO_4). The final mitochondrial pellets were suspended in reaction buffer and adjusted to provide a mitochondrial concentration around 20 mg protein ml^{-1}. Protein assay was performed by the method of Bradford [11]. The condition of the mitochondrial preparations was checked by determination of oxygen consumption and respiratory control with the aid of a fiber optic oxygen monitoring system. Preparations that did not meet the criteria of no detectable endogenous respiration and a succinate-stimulated respiratory control ratio >2.5 were generally excluded from the studies.

2.2. Isolation of mitoplasts

Liver mitoplasts were prepared as described by Ye et al. [12] and Greenawalt [13]. The mitochondria were isolated in buffer medium containing 70 mM sucrose, 220 mM mannitol, 2 mM HEPES and 0.25% BSA, pH 7.35. The mitochondrial suspension was adjusted to 50 mg protein ml^{-1} and treated with 0.6% digitonin by stirring on ice for 15 min. The suspension was centrifuged at 12 000× *g*, and the resulting mitoplast pellets were washed once and re-suspended in reaction buffer.

2.3. Zinc uptake assay

Zinc transport was determined by ^{65}Zn uptake in mitochondria. All ^{65}Zn solutions were dissolved in medium containing 250 mM sucrose and 10 mM HEPES, pH 7.35. Generally 75 μl mitochondria suspension containing 250 μg mitochondrial protein was added to 75 μl reaction buffer containing ^{65}Zn in a 250-μl microfuge tube. After an appropriate incubation time at 37 °C, the reaction tubes were loaded on a Brandel harvesting system (Brandel, Gaithersburg, MD, USA), rapidly aspirated onto filter discs and immediately washed with 20 ml cold isolation buffer. The filters containing the mitochondria were placed into vials containing liquid scintillation cocktail, and the ^{65}Zn was counted in a liquid scintillation counter. The same protocol was used for ^{14}C assay. Generally, the Zn-Ligand solutions were prepared by the addition of the ligand to the stock $ZnCl_2$ solution containing ^{65}Zn to provide a Zn/Ligand molar ratio of 1:3. For total zinc accumulation the mitochondria were digested and the zinc content was determined by atomic absorption as previously described [14].

2.4. Statistics analysis

Zinc accumulation and kinetic experiments were repeated two or three times to ensure the reproducibility of the results. The data and plots were analyzed by SigmaPlot 8.0 with the Enzyme Kinetics Module. The representative results are presented.

3. Results

3.1. Studies with prostate mitochondria

In these initial studies, zinc uptake rates were determined with the following zinc substrates: $ZnCl_2$ as a

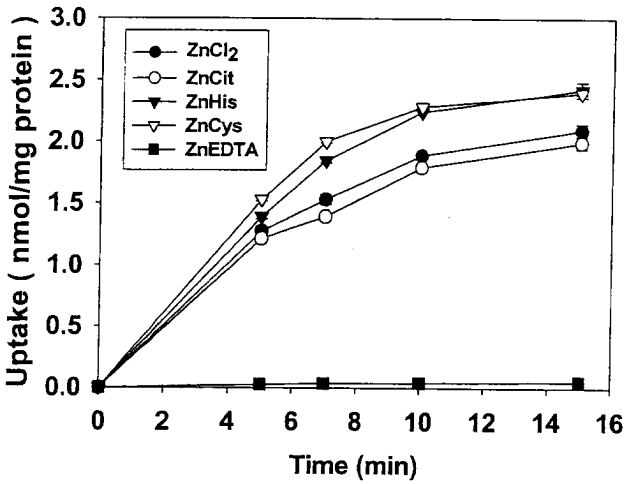

Fig. 1. Time course of ^{65}Zn uptake from $ZnCl_2$, Zn-Citrate, Zn-Histidine, Zn-Cysteine and Zn-EDTA by VP mitochondria. Substrates contained 20 μM $ZnCl_2$ and 60 μM ligand. Incubation at 37 °C.

source of free Zn^{2+} ions; zinc citrate (Zn-Cit), zinc aspartate (Zn-Asp), zinc histidine (Zn-His), and zinc cysteine (Zn-Cys) as potential zinc-transportable Zn-Ligands; and Zn-EDTA and Zn-EGTA as low molecular weight sources of tightly bound zinc. Zn-Cit was selected because it is an important zinc ligand form in prostate cells. Zn-Asp was selected because cellular aspartate concentration, like citrate, is high in prostate cells [15]. Fig. 1 shows a time-course study of zinc uptake by prostate mitochondria. The uptake of ^{65}Zn from 20 μM $ZnCl_2$ (i.e. 20 μM free Zn^{2+} ion) was compared with zinc uptake from Zn-Cit, Zn-His, Zn-Cys and Zn-EDTA. The concentration of total zinc was 20 μM and the ligand was 60 μM. The estimated free Zn^{2+} ion concentration for Zn-Cit is ~4 μM, and is negligible for Zn-EDTA. The results demonstrate that zinc uptake from $ZnCl_2$, Zn-Cit, Zn-His and Zn-Cys were identical and decreased with time over the 15-min uptake period. Evidently, these chelated forms of zinc were as effective as free Zn^{2+} ions as donors for zinc uptake. In contrast, no demonstrable uptake of zinc from Zn-EDTA was evident. This indicates that undissociated Zn-EDTA is not permeable across the mitochondrial inner membrane; and that zinc is not released from EDTA for availability for uptake. These observations are confirmed and extended by studies described below.

Table 1
Kinetic parameters of zinc uptake in rat ventral prostate and liver mitochondria

Mitochondria	Zn	K_m		V_{max}	
		Mean	S.E.	Mean	S.E.
VP	$ZnCl_2$	59.93	9.98	0.63	0.04
	Zn-Asp	54.74	8.53	0.67	0.04
	Zn-Cit	31.14[a,b]	3.23	0.42	0.01
Liver	$ZnCl_2$	80.12	10.71	0.90	0.05
	Zn-Cit	26.52[a]	2.22	0.48[a]	0.01

K_m = μM Zn; V_{max} = nmol Zn min^{-1} mg^{-1} mitochondrial protein.
[a] Zn-Cit vs. $ZnCl_2$, $P<0.01$.
[b] Zn-Cit vs. Zn-Asp, $P<0.01$.

We then determined the relationship of prostate mitochondrial uptake of zinc versus zinc concentration with $ZnCl_2$, Zn-Cit, Zn-Asp and Zn-EDTA as substrates (Fig. 2). Except for Zn-EDTA, the rates of zinc uptake were dependent upon the concentration of zinc. No uptake of zinc was detectable from Zn-EDTA over the range of zinc concentrations that was employed. Zinc uptake from the other substrates exhibited Michaelis–Menten kinetics that demonstrated the existence of a transport process. The K_m (μM zinc) and V_{max} (nmol zinc mg^{-1} mitochondrial protein min^{-1}) values with $ZnCl_2$ and Zn-Asp as substrates were essentially identical (K_m ~55–60; V_{max} ~0.63–0.67; Table 1). The K_m ~31 and V_{max} ~0.42 with Zn-Cit were significantly different from $ZnCl_2$ and Zn-Asp. However at zinc concentrations up to ~50 μM, the uptake rates were essentially the same for all three substrates (Fig. 2A). Because the ratio of ligand to zinc was maintained at 3:1, the free Zn^{2+} ion concentrations for $ZnCl_2$, Zn-Cit, and Zn-Asp preparations were 5–50, 1–10, and 0.05–0.5 μM, respectively (Fig. 2C). Therefore, the uptake of zinc, as in Fig. 1, was independent of the concentration of free Zn^{2+} ion concentration; but was dependent upon the total zinc concentration.

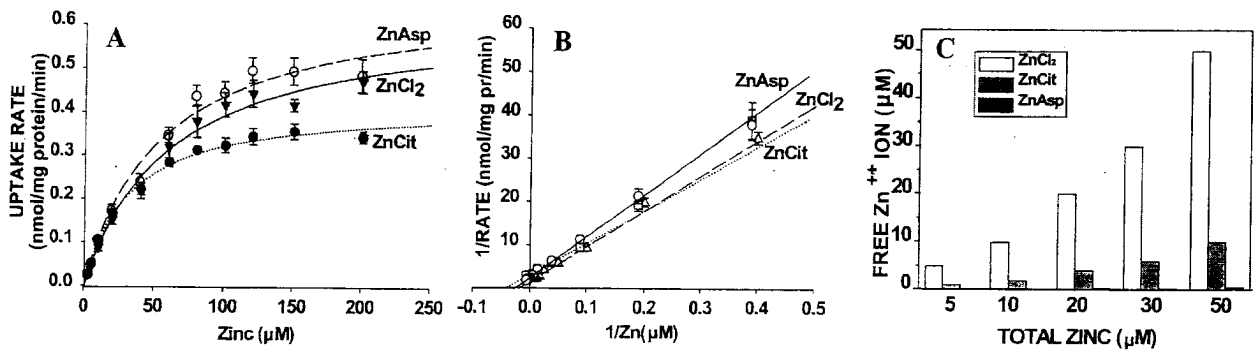

Fig. 2. Zinc uptake from $ZnCl_2$, Zn-Aspartate and Zn-Citrate in rat ventral prostate mitochondria. Uptake was performed at 37 °C, 15 min incubation. For zinc concentrations from 50 to 200 μM, zinc uptake was linear for about 20–30 min. (A) Hyperbolic plot; (B) Lineweaver–Burk plot; (C) free zinc ion concentration.

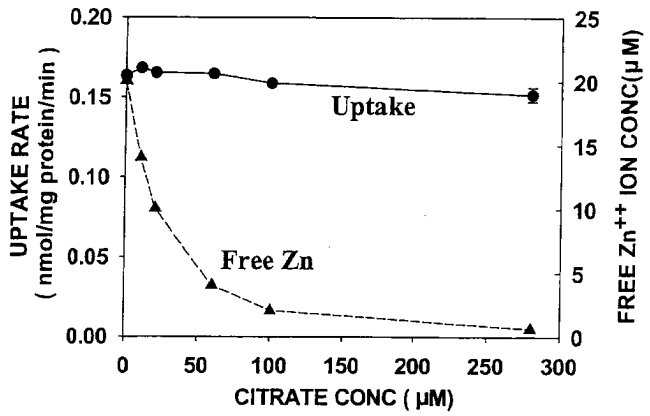

Fig. 3. Effect of added citrate on ^{65}Zn uptake from 20 μM $ZnCl_2$ by rat ventral prostate mitochondria. Uptake was for 15 min at 37 °C.

The effect of varying the citrate/zinc ratio on the uptake of zinc was then determined (Fig. 3). The concentration of zinc ($ZnCl_2$) was maintained at 20 μM. Over the range of citrate/zinc ratios of 0.5:1–15:1 (i.e. the addition of 10–300 μM citrate), the uptake of zinc was the same as in the absence of citrate. Over this range the estimated free Zn^{2+} ion concentration varied from ~11 μM at 0.5:1 ratio to ~1 μM at 10:1 ratio. Consequently the uptake of zinc from Zn-Cit was independent of the free Zn^{2+} ion concentration. Since zinc uptake remained identical to the uptake from $ZnCl_2$ in the absence of any citrate, it is the total zinc (Zn-Cit plus free Zn^{2+} ions) that constitutes the transportable zinc pool.

The possibility existed that the uptake of zinc from Zn-Ligand (i.e. Zn-Cit, Zn-Asp) might result from uptake of the undissociated Zn/Ligand complex. Therefore, we determined the simultaneous uptake of zinc and citrate. Citrate exists predominately as a trianion at the physiological pH range and is virtually impermeable across the mitochondrial membrane in well-maintained coupled mitochondrial preparations. The simultaneous uptake of ^{65}Zn and ^{14}C citrate from Zn-Cit was determined (Fig. 4). The results show that virtually no citrate uptake accompanied the zinc uptake by the mitochondria. In fact the results also demonstrate the integrity of the mitochondria since neither undissociated Zn-Cit (~16 μM) nor free citrate (~44 μM) entered the mitochondria by diffusion or by transport. These results, along with other results presented, indicate that the transport of zinc involves the direct transfer of zinc from Zn-Ligand to the putative zinc transporter; i.e. an inter-molecular zinc transfer that does not necessitate the involvement of a free Zn^{2+} ion donor pool.

The composite studies described above demonstrated the ability of Zn-Cit, Zn-His, Zn-Cys and Zn-Asp, but not Zn-EDTA nor Zn-EGTA (Fig. 1 and 11, described below), to act as effective zinc donors for mitochondrial transport. This indicated that the formation constant (K_f) of Zn-Ligands is an important determinant for the availability of zinc for mitochondrial transport. The log K_f value for each ligand is Zn-Cit ~5, Zn-Asp ~6, Zn-His ~6, Zn-Cys ~10, Zn-EGTA ~12 and Zn-EDTA ~16. Therefore, all the Zn-Ligands with log K_f~10 or lower served as effective donors of zinc for mitochondrial transport. Zn-Ligands with log K_f~12 or higher did not donate zinc for transport. These results indicate that the putative zinc transporter has an effective log K_f~11.

The above observations lead to the conclusion that mitochondrial zinc uptake under the conditions employed was the result of the existence of a transport process; i.e. a putative zinc transporter. The following studies were then conducted to characterize further the transport properties. To obtain some indication of the metal specificity of the zinc transport, the effects of Ca^{2+}, Mg^{2+}, and Cd^{2+} on zinc uptake was determined (Fig. 5). Ca^{2+} and Mg^{2+} at

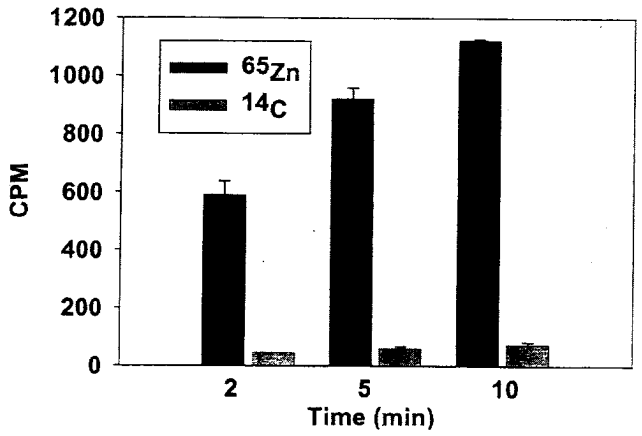

Fig. 4. ^{65}Zn and ^{14}C-citrate uptake from Zn-Citrate at 37 °C. Zn-Citrate was prepared with 20 μM $ZnCl_2$ and 60 μM citrate.

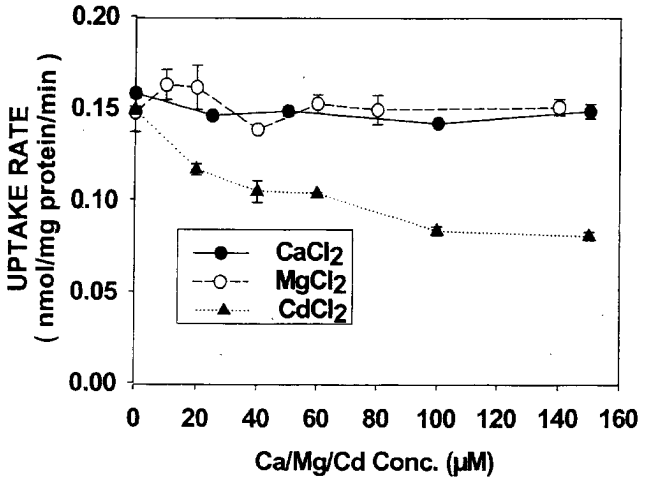

Fig. 5. Divalent cation effects on ^{65}Zn uptake from Zn-Citrate. VP mitochondrial uptake at 37 °C, 15 min incubation. Indicated concentration of $CaCl_2$, $MgCl_2$ and $CdCl_2$ was added to reaction buffer containing 20 μM $ZnCl_2$ and 60 μM citrate.

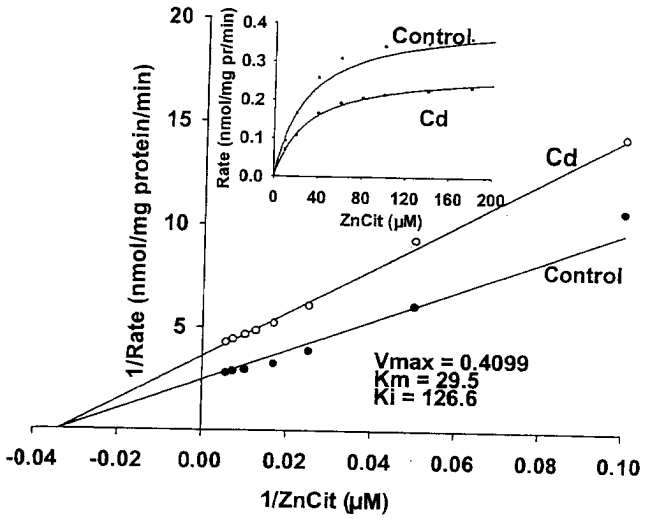

Fig. 6. Cd^{2+} (60 μM) inhibition of zinc uptake from Zn-Citrate. VP mitochondria at 37 °C and 15 min incubation. Lineweaver–Burk plot and hyperbolic plot.

concentrations up to 7–8-fold greater than zinc had no effect on the uptake of zinc from Zn-Cit. The absence of any effect by Ca^{2+} also indicates that the uptake of zinc does not involve a Ca^{2+} ion channel. However, Cd^{2+} at equimolar concentration with zinc exhibited ~20% inhibition of zinc uptake; which increased to a maximum inhibition ~40% by increasing the Cd^{2+} to 5-fold greater than zinc. Lineweaver–Burk plot (Fig. 6) revealed a K_i~ 126 μM Cd^{2+}, and a noncompetitive inhibition. Because some zinc transporters evolved as zinc/ferrous iron transporter (e.g. ZIP transporter family), it was important to determine if Fe^{2+} competed with the mitochondrial import of zinc. To insure that Fe^{2+} was not oxidized, dithionite was also added. Fe^{2+} with or without dithionite had no effect on zinc uptake by mitochondria (results not shown).

The transport studies described above were conducted with non-respiring coupled mitochondrial preparations that contained no added utilizable energy sources. This suggested that the uptake of zinc might be energy-independent. To establish this relationship zinc uptake was determined under conditions that would alter the energy and respiratory state of the mitochondria (Fig. 7A). The addition of succinate alone or with ADP had no effect on the uptake of zinc. Neither the addition of the uncoupling agent dinitrophenol nor the addition of cyanide to inhibit cytochrome oxidase and respiration altered the uptake of zinc. We conducted parallel studies (not shown) of the respiration and terminal oxidation of the mitochondrial preparations to verify the treatment effects.

Consequently, zinc transport is not energy-dependent. In addition, over the range of pH 6.0–8.0, there occurred no significant change in the zinc uptake rate (Fig. 7B). Therefore, the collective results of these studies strongly indicate that the mitochondrial uptake of zinc is the result of a facilitative zinc transporter and not an active transport process.

3.2. Studies with liver mitochondria

The studies described above establish the existence of a zinc transport process (putative zinc uptake transporter) involved in the mitochondrial uptake and accumulation of zinc in prostate cells. Whether or not this zinc transport process is specific and unique to prostate mitochondria

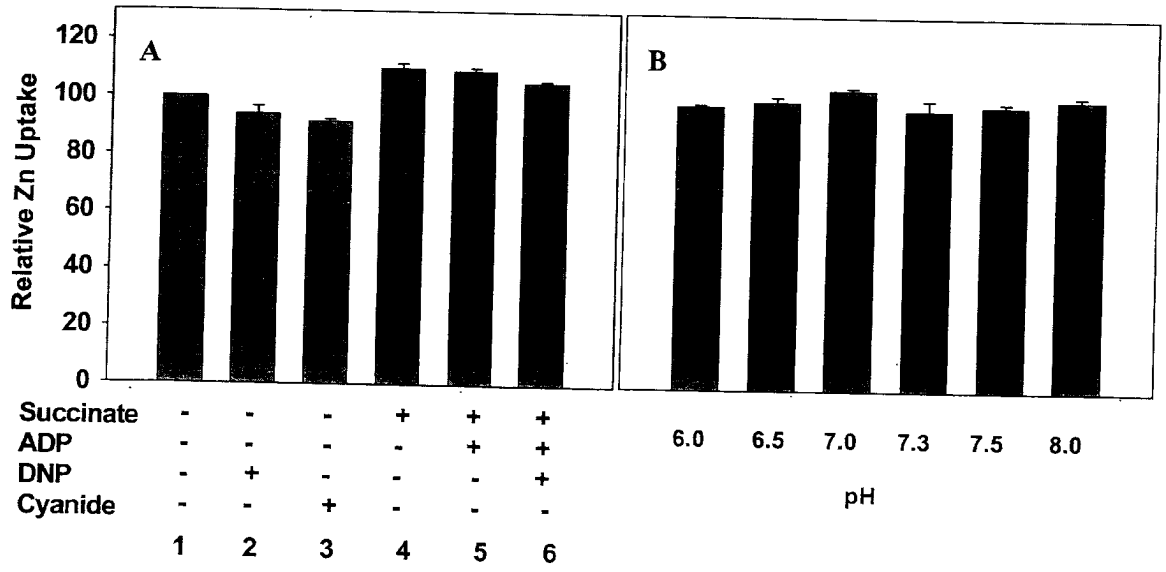

Fig. 7. pH (B) and energy (A) effect on ^{65}Zn uptake from Zn-Citrate by VP mitochondria: 15-min incubation at 37 °C. Zn-Citrate preparation contained 20 μM $ZnCl_2$ and 60 μM citrate. Succinate 8 mM, ATP 1 mM, cyanide 2.5 mM and DNP 0.2 mM.

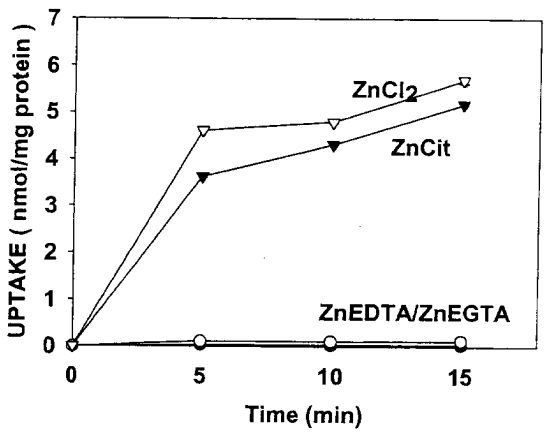

Fig. 8. Time course of ^{65}Zn uptake from Zn-EDTA, Zn-EGTA, Zn-Citrate and $ZnCl_2$ by rat liver mitochondria. Substrates contained 50 μM $ZnCl_2$ and 150 μM ligand.

needed to be addressed. Consequently, some zinc uptake studies were conducted with liver mitochondria. Fig. 8 reveals that liver mitochondria, like prostate mitochondria (Fig. 1), exhibited zinc uptake from Zn-Cit as well as $ZnCl_2$, but not from Zn-EDTA or Zn-EGTA. Lineweaver–Burk analysis (Fig. 9) of zinc uptake results in K_m ~80 μM and V_{max} ~0.90 nmol zinc uptake min^{-1} mg^{-1} mitochondrial protein for $ZnCl_2$; and K_m ~27 and V_{max} ~0.48 for Zn-Cit. It is interesting to note that the K_m and V_{max} values of liver and prostate mitochondria are quite similar for Zn-Cit (Table 1); but the values are highly dissimilar for $ZnCl_2$. However at zinc concentrations up to ~50 μM, liver mitochondria zinc uptake from $ZnCl_2$ and Zn-Cit was identical (Fig. 9); as was also the case for prostate mitochondria (Fig. 2). These studies demonstrate that the uptake of zinc by liver mitochondria is not dependent upon a free Zn^{2+} ion pool; and that Zn-Ligands provide an effective zinc donor source for zinc uptake. Therefore, it is evident that liver mitochondria, like prostate mitochondria, also contain a zinc uptake transport process (i.e. a zinc transporter).

It became essential to establish that the zinc transport process as determined by the ^{65}Zn uptake studies was associated with an increased net accumulation of zinc in the mitochondria. Therefore, we determined, under the conditions employed for the ^{65}Zn experiments, the level of total zinc in liver mitochondria after incubation with $ZnCl_2$, Zn-Cit, Zn-Cys, and Zn-EDTA (Fig. 10A). A rapid accumulation of high zinc levels was evident within 2 min and reached a peak by 10–15 min with $ZnCl_2$, Zn-Cit and Zn-Cys as the sources of zinc. With Zn-EDTA, no zinc accumulation resulted. These results parallel the ^{65}Zn uptake studies. Importantly, the net accumulation of zinc is not dependent upon a pool of free zinc since free zinc ions are negligible in the presence of cysteine. The absence of accumulation with Zn-EDTA further indicates that the relative zinc-binding affinity for Zn-Ligand and putative transporter is a key factor in the mitochondrial transport process. The absence of accumulation with Zn-EDTA is also evidence of the integrity of the mitochondrial preparations. A subsequent study (Fig. 10B) demonstrates that prostate and liver mitochondria accumulate similar levels of total zinc. It is noteworthy that the maximal total zinc accumulation of about 5–6 nmol mg^{-1} protein at 10–15 min approximates the calculated zinc accumulation from the ^{65}Zn uptake studies. When adjusted for 50 μM zinc in medium, the estimated total zinc accumulation represented in Figs. 1, 8, and 11 is about 5–7 nmol mg^{-1} protein. This confirms that the uptake of ^{65}Zn was retained and accumulated in the mitochondria.

Since low molecular weight solutes such as the Zn-Ligands employed in these studies would be permeable across the mitochondrial outer membrane, the likely site for the putative zinc transporter would be the mitochondrial inner membrane. To investigate this likelihood mitoplasts were prepared from liver mitochondria to eliminate the outer membrane. Zinc uptake by the mitoplasts was compared with the in tact mitochondria (Fig. 11). Zinc uptake from $ZnCl_2$ and Zn-Cit by the mitoplast paralleled the uptake by the intact mitochondria. The absence of any zinc uptake from Zn-EDTA and Zn-EGTA demonstrates that the integrity of the inner membrane was retained in the

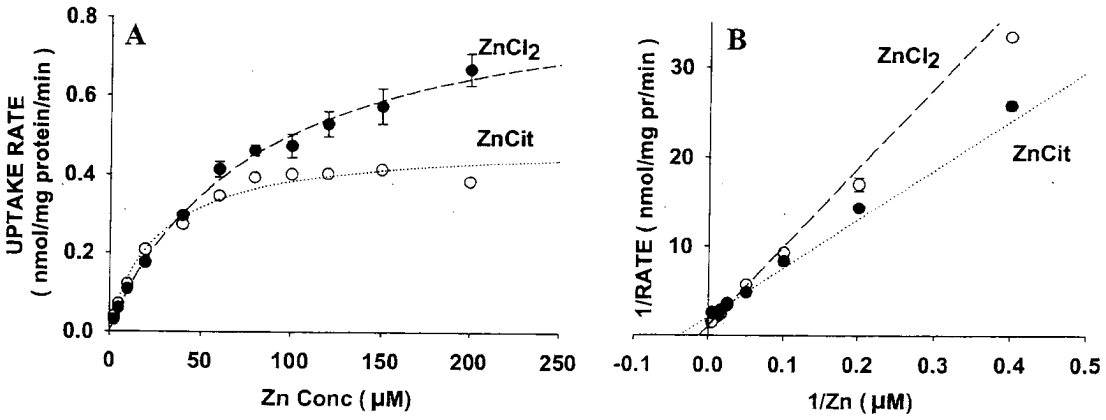

Fig. 9. Zinc uptake from $ZnCl_2$ and Zn-Citrate in rat liver mitochondria: 15 min incubation at 37 °C.

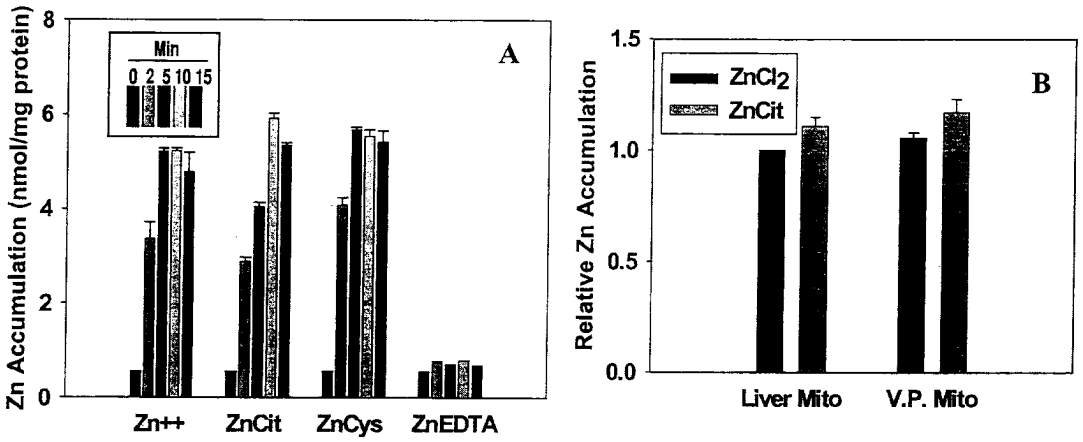

Fig. 10. Total zinc accumulation in mitochondria. Zinc concentration was 30 μM and ligands were 90 μM. After incubation, reaction tubes were rapidly centrifuged and mitochondrial pellets were digested and assayed for total zinc by atomic absorption. (A) Zinc accumulation by liver mitochondria. (B) Comparison of total zinc accumulation in liver and prostate mitochondria.

mitoplast preparation. It is also significant that the mitoplast uptake of zinc from Zn-Cit was slightly less than from $ZnCl_2$; which is a characteristic that occurred in all of the in tact mitochondrial preparations from prostate and from liver. These results support the expectation that the putative zinc uptake transporter is associated with the mitochondrial inner membrane.

4. Discussion

These studies reveal that zinc uptake and accumulation by prostate and liver mitochondria occurs via a zinc transport process. The uptake of zinc is saturable, energy-independent, and exhibits kinetic characteristics that are representative of the existence of a facilitative zinc transporter. The transporter appears to be associated with the mitochondrial inner membrane. The putative transporter exhibits a high specificity for zinc since neither Ca^{2+}, nor Mg^{2+}, nor low concentration of Cd^{2+} altered the uptake of zinc. To our knowledge this is the first identification of a specific mitochondrial zinc uptake transport process in mammalian cells. It will be important to identify the putative transporter that is responsible for this zinc transport activity. To date, no genetic or proteomic identification of such a transporter has been established.

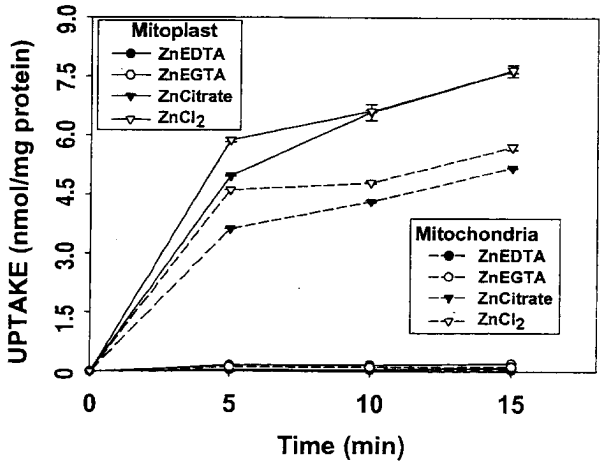

Fig. 11. Time course of zinc uptake from Zn-EDTA, Zn-EGTA, Zn-Citrate and $ZnCl_2$ in rat liver mitochondria vs. mitoplasts at 37 °C. Substrates contained 50 μM $ZnCl_2$ and 150 μM ligand.

This study reveals that the availability of free Zn^{2+} ions is not a required source of donor zinc for transport and accumulation in mitochondria. Zinc derived from Zn-Ligands was directly transferred to the transporter, which provides the mechanism for zinc uptake by the mitochondria. This is further verified by the mitochondrial transport of zinc from Zn-Ligand without an accompanying uptake of the ligand. Also, the absence of zinc uptake from either Zn-EDTA or Zn-EGTA indicates that undissociated low molecular weight Zn-Ligands are not permeable or transportable across the mitochondrial inner membrane. An important determinant of the availability of zinc for transport is the formation constant (K_f) of the Zn-Ligand; i.e. the binding affinity of ligand for zinc. The effective log K_f of the transporter is estimated to be ~11 since Zn-Ligands with log K_f<11 were equally effective zinc donors available for transport, and Zn-Ligands with log K_f>11 were ineffective zinc donors.

The effectiveness of Zn-Ligands to donate zinc for mitochondrial import in prostate cells is consistent with the fact that the free Zn^{2+} ion concentration of cytosol is in the nanomolar to femtomolar range; and, therefore, does not provide a sufficient zinc donor pool for mitochondrial uptake leading to zinc accumulation. Moreover the K_m for zinc uptake from $ZnCl_2$ is ~60 μM zinc, which is 1000-fold or greater than the cytosolic concentration of free Zn^{2+} ions. In contrast the effective Zn-Ligands (e.g. amino acids, citrate) likely constitute a zinc pool in the micromo-

lar range that would be functional at the K_m range exhibited by the prostate and liver mitochondrial transport.

These studies show that both prostate and liver mitochondria are similarly capable of importing and accumulating zinc from Zn-Ligands. Moreover, both exhibit similar values of maximal zinc accumulation, i.e. about 2–7 nmol Zn mg^{-1} protein in the presence of zinc in the range of 20–50 μM. The in situ level of zinc in ventral prostate cells approximates 2 nmol mg^{-1} protein [14], which is virtually identical to the experimental value obtained in this study. Liver mitochondria in situ contains only about 0.6 nmol Zn mg^{-1} protein although the capacity for zinc accumulation is similar to prostate mitochondria [12,14]. This difference is consistent with our concept that the higher cytosolic concentration of zinc-transportable Zn-Ligands (particularly Zn-Cit) in prostate cells likely accounts for the characteristically higher zinc accumulation of prostate mitochondria compared to other cells. The similarity of the experimental level of zinc accumulation to the in situ mitochondrial zinc level provides additional support that the mitochondrial zinc uptake transport process identified in this study most likely is functionally operational in situ.

Despite the fact that zinc uptake and accumulation in mitochondria has long been known to be an important cellular functional relationship, few reported studies have addressed the issue of the mechanisms and processes of zinc entry into mitochondria. Most of the early studies of zinc uptake and its effects on mitochondria employed free Zn^{2+} ions at concentrations that are now known to be highly unphysiological. Brierely and Knight [16] reported that heart mitochondria accumulated zinc by energy-dependent and by passive processes depending upon the conditions employed; but free Zn^{2+} in the range of 100–500 μM was employed in those studies. It has been suggested that free Zn^{2+} ions enter the mitochondria via the calcium uniporter [17]. However those studies were also conducted with μM concentrations of free Zn^{2+} ions, with no evidence that Zn^{2+} at levels more representative of cytosol (10^{-9} M or lower) would successfully traverse the ion channel resulting in sufficient accumulation of intramitochondrial zinc. Also no direct measurements of mitochondrial uptake of zinc were provided. It is notable that Ye et al. [12] reported that metallothionein was an important chaperone for the delivery of cytosolic zinc to liver mitochondria. They showed that cytosolic metallothionein enters the intermembrane space where it relinquishes zinc that inhibits respiratory components at that location. Although they report that metallothionein does not enter into the mitochodrial matrix, no information was presented regarding the possible accumulation of zinc in the matrix. Our studies show that Zn-Cys (log K_f~10) is an effective zinc donor for the mitochondrial transport of zinc. Metallothionein also has a log K_f~10, and could possibly serves as a zinc donor for the zinc uptake transporter. We are making preparations to investigate the potentially important role of metallothionein in the exchange of zinc with the putative mitochondrial transporter for the uptake of zinc.

Our findings and this discussion are not intended to eliminate the involvement of other mitochondrial zinc import mechanisms that might exist. The process that we have now identified is operational under conditions that mimic the likely in situ availability of transportable zinc forms that exist in cytosol, especially in regard to the unique zinc relationships of prostate cells. Clearly much additional study of the mitochondrial zinc-uptake transport process and the putative transporter that we have described is essential. This initial report establishes the existence of such a mitochondrial transporter and provides the basis for further research.

5. Abbreviations

ADP	adenosine 5′-diphosphate
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
EGTA,	ethylene-glycol-bis(β-aminoethlyether)*N,N,N,N*-tetraacetic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

Acknowledgements

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